UNIVERSITY OF ALBERTA

TRANSLATION INITIATION FACTORS eIF4B AND eIF4H STIMULATE THE RIBONUCLEASE ACTIVITY OF THE VIRION HOST SHUTOFF PROTEIN OF HERPES SIMPLEX VIRUS 1

by

ROSALYN C. DOEPKER

©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Virology.

Department of Medical Microbiology and Immunology

Edmonton, Alberta

Spring 2004

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 0-612-96466-3
Our file Notre référence
ISBN: 0-612-96466-3

The author has granted a non-exclusive license allowing the Library and Archives Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou aturement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

ABSTRACT

The virion host shutoff protein (vhs) of herpes simplex virus triggers the shutoff of host protein synthesis and degradation of mRNAs. Vhs specifically targets mRNAs and binds to the translation initiation factor eIF4H. A complex of vhs/eIF4H has ribonuclease activity in the absence of any other host or viral factors. However, the role of eIF4H and other host factors in the activity and mRNA targeting of vhs has not yet been directly examined. Yeast extracts expressing vhs were previously reported to lack nuclease activity, however, we observed detectable nuclease activity which did not target to the EMCV internal ribosome entry site (IRES), in the absence of mammalian factors. The activity of vhs produced in yeast was strongly stimulated by RRL, eIF4H or its sequence paralogue, eIF4B, and only RRL was able to reconstitute IRES-directed targeting. These results demonstrate that additional mammalian factors are required for targeting to the EMCV IRES.

ACKNOWLEDGEMENTS

The completion of my M.Sc. degree and the production of this thesis could not have been accomplished without the help of many people. First of all, I would like to thank GOD, who has been with me always even when I was not *aware*. Secondly, I would like to thank my supervisor Dr. Jim Smiley, whose door was always open if you wanted to talk. He provided a *phenomenal cosmic* laboratory, with an itty bitty office space ☺.

I also have to thank all of the members of the Smiley Lab, without whom I would have been lost and life would just not have been as much fun. Thanks go to Kim Ellison, Holly Saffran, Rob Maranchuck, Leslie Shai, Henrietta Bohr, Wei-li Hsu, Sarah Richart, Rebecca Minaker, and Graeme Quest. A special thank you to Kim and Holly for great wisdom and technical advice, to Rob for all the pain you had to go through with my figures, and to Leslie, Rebecca and Graeme for all the fun we had.

To Henrietta , I offer you my heartfelt thanks for all of the crazy times we had, the outrageous conversation topics, for support when I felt like Buffy in Gone, for motivation in the lab and for generally being a great friend. I will miss you very much.

There are a few other good friends who have to be mentioned, namely Lori Buhr and Danielle Lutes. Lori, I will miss our monthly trips to Ikea and Danielle, I am so glad we had the chance become better friends and our trip to Germany will be something I will never forget. The list would not be complete without also thanking two other 'friends' who helped me get through my darkest hours of thesis writing – Buffy and Angel.

I also have to thank the Alberta Heritage Foundation for Medical Research who have generously supported me for three years.

My family has been a great support to me and I would like to thank my Mom and Dad, Beatrice and Wally Doepker, and my two fun-loving sisters, Andrea and Jocelyn Doepker.

Last but certainly not least, I have to thank the man who is my love and my strength, Scott Bunnell. You complete me and I would be lost without you, what more is there to say?

TABLE OF CONTENTS

CHAPTER 1 : INTRODUCTION ____ **1**

1.1 Introduction to Herpesviruses ____ 1

1.2 Overview of HSV Infection ____ 2

1.3 Epidemiology and Clinical Manifestations of HSV Infection ____ 3

1.4 HSV Virion ____ 3

1.5 HSV Lytic Infection ____ 5

1.5.1 *Virus Attachment and Entry* ____ 5

1.5.2 *Immediate–Early Gene Expression* ____ 7

1.5.3 *ICP0* ____ 10

1.5.4 *ICP4* ____ 12

1.5.5 *ICP22/Us1.5* ____ 15

1.5.6 *ICP27* ____ 17

1.5.7 *ICP47* ____ 20

1.5.8 *Early Gene Expression and Viral DNA Replication* ____ 21

1.5.9 *Late Gene Expression, Virion Assembly and Egress* ____ 23

1.6 Virion Host Shutoff ____ 26

1.7 Translation Initiation ____ 34

1.8 Thesis Rational ____ 36

CHAPTER 2 : MATERIALS AND METHODS ____ **40**

2.1 Plasmids ____ 40

2.2 Bacterial Strains and Growth Conditions ____ 42

2.3 Yeast Strain, Growth Conditions, and Yeast Transformation ____ 43

2.4 Preparation of Yeast Extracts for the Vhs Activity Assay ____ 43

2.5 Protein Expression and Purification ____ 43

2.6 Western Blot ____ 45

2.7 *In Vitro* Transcription and RNA Labeling ____ 45

2.8 *In Vitro* Translation 46

2.9 Vhs Activity Assay 47

2.10 Agarose Gel Electrophoresis and Northern Blot Analysis 47

2.11 Far Western Analysis 48

2.12 Helicase Unwinding Assay 49

CHAPTER 3 : RESULTS 55

3.1 Expression of vhs in *S. cerevisiae* Induces a Ribonuclease Activity That is Stimulated by One or More Mammalian Factors 55

3.2 eIF4H Stimulates the Endoribonuclease Activity of vhs Produced in Yeast 57

3.3 Effects of RRL and eIF4H on Targeting of vhs Activity to the EMCV IRES. 60

3.4 eIF4B Interacts with vhs 62

3.5 eIF4B Stimulates the Endoribonuclease Activity of vhs Expressed in Yeast 64

CHAPTER 4 : DISCUSSION 91

CHAPTER 5 : REFERENCES 106

LIST OF FIGURES

Figure 1.1: Mechanism of translation initiation. 39

Figure 2.1: Diagram of the cloning strategy to create the eIF4H and eIF4Hi expression vectors. 52

Figure 2.2: Diagram of the cloning strategy to create the eIF4A and eIF4B expression vectors. 54

Figure 3.1: Extracts of yeast expressing 2.1vhs contain endoribonuclease activity that is enhanced by one or more mammalian cofactors. 68

Figure 3.2: Comparison of the nuclease activity of 2.1vhs yeast extracts on pCITE-1 and SRPα RNA substrates. 70

Figure 3.3: Analysis of purified proteins. 72

Figure 3.4: The enhancement of the RNase activity of vhs expressed in yeast is specific to eIF4H. 74

Figure 3.5: eIF4H stimulates the endoribonuclease activity of vhs produced in yeast. 76

Figure 3.6: eIF4H does not restore efficient IRES-directed targeting to yeast-expressed vhs. 78

Figure 3.7: Region of greatest similarity between eIF4H and eIF4B. 80

Figure 3.8: eIF4B interacts *in vitro* with vhs. 82

Figure 3.9: eIF4B stimulates the endoribonuclease activity of the vhs protein produced in yeast. 84

Figure 3.10: eIF4A does not enhance the activity of yeast extracts containing vhs at lower concentrations. 86

Figure 3.11: Helicase activity of eIF4A. 88

Figure 3.12: eIF4A and eIF4B do not restore efficient IRES-directed targeting to yeast expressing vhs. 90

LIST OF ABBREVIATIONS

C	Carboxy
cdc	Cyclin dependent kinase
CTD	Carboxy-terminal domain
CTL	Cytotoxic T lymphocyte
E	Early
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
eIF	Eukaryotic initiation factor
EMCV	Encephalomyocarditis virus
EV	Empty vector
FEN-1	5' flap endonuclease/5'-3' exonulcease
γ1	Leaky-late
γ2	True-late
g	Glycoprotein
HCF	Host cell factor
HCMV	Cytomegalovirus
HHV-6	Human herpesvirus 6
HHV-7	Human herpesvirus 7
HHV-8	Human herpesvirus 8
HMG1	High mobility group factor 1
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
Hve	Herpesvirus entry mediator
ICP	Infected cell polypeptide
IE	Immediate-early
IFN-α	Alpha interferon
IFN-β	Beta interferon
Inr	Initiator sequence
IRES	Internal ribosome entry site
kb	Kilobases
kDa	Kilodalton
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Late
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
N	Amino
ND10	Nuclear domain 10
NES	Nuclear export signal
NLS	Nuclear localization signal

nt	Nucleotides
Oct-1	Octamer transcription factor 1
PABP	Poly(A) binding protein
PML	Promyclocytic leukemia protein
PTB	Pyrimidine tract-binding protein
RNAP II	RNA polymerase II
RNase	Ribonuclease
RRL	Rabbit reticulocyte lysate
RRLvhs	RRL containing pre-translated vhs
RRM	RNA recognition motif
SRPα	Signal recognition particle alpha-subunit
TAF	TBP-associated factor
TBP	TATA-binding protein
TNF	Tumour necrosis factor
ts	Temperature-sensitive
UV	Ultra-violet
VIC	VP16 induced complex
vhs	virion-associated host shutoff
vhs	virion-associated host shutoff protein
VP16	Virion protein 16
VZV	Varicella-zoster virus

CHAPTER 1 : INTRODUCTION

1.1 INTRODUCTION TO HERPESVIRUSES

Members of the *Herpesviridae* family all share a common virion morphology. A herpesvirus virion consists of four distinct components: an electron dense core containing the linear double-stranded DNA; an icosadeltahedral capsid; a tegument, which is an amorphous, proteinacious layer surrounding the capsid; and a glycoprotein containing envelope (Roizman, 1996). Viruses in the *Herpesviridae* family also share a number of biological properties: they encode a large number of enzymes; DNA replication and assembly of capsids occurs in the nucleus; lytic infection results in the destruction of the infected cell; and they establish lifelong latent infections in their natural hosts (Roizman, 1996).

There are over 100 different herpesviruses that have been identified to date, with eight viruses that infect humans. The family *Herpesviridae* has been divided into three subfamilies: the *alphaherpesvirinae*, the *betaherpesvirinae*, and the *gammaherpesvirinae* (reviewed in (Roizman, 1996)). The viruses in the subfamily *alphaherpesvirinae* have a broad host range, a short production cycle, rapidly infect and kill cells, and establish latency in sensory neurons. This subfamily contains the first human herpesviruses to be recognized, the human herpes simplex viruses type 1 and 2 (HSV-1 and HSV-2). The human varicella-zostervirus (VZV) is also a member of this subfamily. The viruses belonging to the subfamily *betaherpesvirinae* generally have a restricted host range, a long reproductive cycle, grow slowly in culture, often cause infected cells to become enlarged and establish latent infections in tissues such as secretory glands and hematopoietic progenitor cells. The human herpesviruses belonging to this subfamily are the human cytomegalovirus (HCMV) and human herpesviruses 6 (HHV-6) and 7 (HHV-7). In the subfamily *gammaherpesvirinae,* viruses have a narrow host range, can replicate in epithelial cells, establish latency in T or B

lymphocytes, and can immortalize infected lymphocytes. This subfamily includes the Epstein-Barr virus (EBV) and the Karposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8).

1.2 Overview of HSV Infection

The pathogenesis of HSV infection of the human host can be divided into four stages. The first stage is characterized by the primary infection which requires intimate personal contact of a seronegative person with an infected secretion (reviewed in (Roizman & Knipe, 2001, Whitley, 1996)). HSV-1 is generally spread by oral contact or saliva and HSV-2 is usually spread by genital contact or vaginal secretions. Infection is initiated when the virus comes into contact with mucosal epithelium (predominately oral mucosal tissue for HSV-1 and genital mucosal tissue for HSV-2) or breaks in the skin. The virus then productively replicates at the site of the infection. The second phase of infection is characterized by the latent infection of neurons. At the site of the primary infection the virus enters the nerve endings of the innervating neurons and the nucleocapsid travels by retrograde axonal transport to the nucleus of the sensory ganglia (primarily the trigeminal ganglion for HSV-1 and the sacral ganglia for HSV-2). The virus undergoes a period of transient replication after which a latent state is established. The viral genome circularizes and remains in an episomal state in the majority of infected neurons for the lifetime of the individual. The third stage is the reactivation of the virus. The virus may be activated by a variety of stimuli such as, physical or emotional stress, exposure to ultraviolet light, tissue damage, fever, immune suppression, and axonal injury. The virus undergoes a least a limited productive cycle and capsids are carried by anterograde transport to the cells at or near the site of the initial infection. The fourth stage of HSV infection is characterized by recurrent infection. The reactivated infection involves the same area as the original infection. The virus replicates in the mucosal tissue causing the shedding of virus. Reactivation can be asymptomatic or lead to the formation of skin vesicles or mucosal ulcers.

1.3 Epidemiology and Clinical Manifestations of HSV Infection

As mentioned above, HSV is transmitted from infected to susceptible individuals by close personal contact. HSV-1 infection is common world wide with serological studies indicating that 70 – 95 % of the population is infected with HSV-1, depending on the country (Whitley & Gnann, 1993). Although reciprocal infections can occur, HSV-1 is generally associated with infections above the waist and HSV-2 with infections below the waist. Primary HSV-1 infection usually occurs before the age of 5 and is often asymptomatic (Whitley, 1996). Herpetic gingivostomatitis is the common clinical manifestation in younger children, where as, in young adults primary HSV-1 infection often results in herpes pharyngitis and a mononucleosis-like syndrome (Glezen *et al.*, 1975, McMillan *et al.*, 1993). The most common clinical manifestation of recurrent HSV-1 infection are vesicular lesions or more commonly known as cold sores, which are estimated to affect about 33 % of adults (Friedman *et al.*, 1977, Ship *et al.*, 1967, Ship *et al.*, 1977). Other clinical syndromes caused by HSV-1 include: herpetic keratitis, herpetic whitlow, eczema herpeticum and herpes encephalitis (Simmons, 2002).

HSV-2 spread is primarily through sexual contact, and as such initial infection usually occurs after the onset of sexual activity (Whitley, 1996). Infection with HSV-2 is quite prevalent, with seropositivity to HSV-2 ranging from 5-25 % in western countries (Smith & Robinson, 2002). Recurrent HSV-2 infection causes genital herpes and very rarely HSV meningitis. HSV-2 can also cause devastating neonatal infections (disease localized to the skin, eye and mouth, encephalitis and disseminated infection involving multiple organs) (Whitley, 1996), with HSV-2 infection accounting for 35 % of neonatal encephalitis (Ferrante *et al.*, 2000).

1.4 HSV Virion

The HSV virion consists of four components: a DNA core, a capsid, a tegument surrounding the capsid and an envelope (Roizman, 1996). The HSV DNA is double-stranded, linear and wrapped in the form of a toroid or a spool (Zhou *et al.*, 1999). In the nucleus of infected cells, the HSV genome remains linear (Jackson & DeLuca, 2003). The viral genome of HSV-1 strain 17 is just over 152 kilobase pairs long, with a GC content of 68 % for HSV-1 and 69 % for HSV-2 (Becker *et al.*, 1968). It can be divided into two covalently linked components: the long and short regions. Each component contains unique sequences that are flanked by inverted repeats. The repeated sequences bracketing the unique long (U_L) sequence are designated ab and b' a', and the repeated sequences bracketing the unique short (U_S) sequence are designated a'c' and ca (McGeoch *et al.*, 1988, McGeoch *et al.*, 1985). Through recombination, the inverted repeats allow the long and short components to invert relative to one another to yield four linear isofoms. In an infected cell, the HSV genome exists in equimolar concentrations of all four isomers (Delius & Clements, 1976, Hayward *et al.*, 1975). The HSV genome encodes about 90 unique proteins whose genes are transcribed from both DNA stands. Those genes which are located in the inverted repeats are present in two copies (Roizman & Knipe, 2001). For the most part, each gene has its own promoter, although some transcripts share 3' ends. The HSV virion contains 30 known and 10 suspected virus proteins distributed in the capsid, tegument and envelope (Roizman & Knipe, 2001).

The icosadeltahedral capsid is composed of 162 capsomers. Capsid assembly requires seven proteins which are encoded by six genes: UL18 (VP23), UL19 (VP5), UL26 (VP21 and VP24), UL 26.5 (VP22a), UL35 (VP26), and UL38 (VP19C) (Tatman *et al.*, 1994). The UL26 protein product has protease activity which cleaves autoproteolytically to create VP21 and VP24, and also cleaves pre-VP22a into VP22a (Homa & Brown, 1997). The proteins VP5, VP19C, VP23 and VP26 are the structural components of the outer capsid

shell and VP21, VP22a and VP24 form the scaffold structure around which the capsid shell forms (Homa & Brown, 1997).

The tegument is designated as the proteinacious layer between the capsid and the envelope. It is largely unstructured and contains many proteins required during the early events of infection (Zhou *et al.*, 1999). Some of these proteins include VP16, virion host shutoff (vhs) protein, VP22, VP13/14, VP11/12, and VP1/2 (Roizman & Knipe, 2001). VP16 is important for its transactivating activity on immediate-early (IE) gene promoters and vhs is required for the shutoff of host protein synthesis. Both proteins will be discussed in more detail below.

The envelope consists of a lipid bilayer with protruding glycoprotein spikes. There are at least 10 glycoproteins on the surface of the virion. They are gB, gC,gD, gE, gG, gH, gI, gK, gL and gM (Roizman & Knipe, 2001). Non-glycosylated membrane proteins include the products of UL20 and US9, and possibly UL24, UL43 and UL34 (Roizman & Knipe, 2001).

1.5 HSV Lytic Infection

The HSV lytic infection proceeds though the following stages: virus attachment and entry, capsid transport to the nucleus and release of the viral genome, expression of the IE genes, expression of the early (E) genes and viral DNA replication, and expression of the late (L) genes and virion assembly and egress. Each step will be discussed in more detail below.

1.5.1 Virus Attachment and Entry

HSV-1 meditates entry into the host cell via attachment at the cell surface followed by fusion between the viral envelope and the host cell membrane. There are five viral glycoproteins which aid in viral entry: gB, gC, gD, gH and gL (Spear, 1993). With the exception of gC, all are essential for viral entry, as mutant virions lacking one of the glycoproteins can bind but not enter cells

(reviewed in (Shukla & Spear, 2001)). Virions which lack gC show an impairment in adsorption, and a delay in penetration of cells, however, bound virus can still enter cells and initiate infection (Herold *et al.*, 1991), where as virions lacking both gB and gC are severely impaired for binding to cells (Herold *et al.*, 1994). The first step in entry of HSV into cells is the initial interaction of the HSV membrane glycoproteins gB or gC with the heparan sulfate chains on cell surface proteoglycans (Shieh *et al.*, 1992, WuDunn & Spear, 1989). Both gB and gC bind heparan sulfate (Herold *et al.*, 1991, Tal-Singer *et al.*, 1995, Williams & Straus, 1997) and soluble gC can inhibit the virus from binding to cells (Svennerholm *et al.*, 1991, Tal-Singer *et al.*, 1995). However, cells which do not express heparan sulfate but do express other glycoaminoglycans can also be infected, although with decreased efficiency and glycoprotein gD appears to play a role in the infection of this cell line (Banfield *et al.*, 1995).

The second step in attachment involves the binding of the viral ligand gD to one of several entry receptors. gD can independently bind entry receptors belonging to four classes of molecules (Spear, 1993). The first is the herpes virus entry mediator (HveA), which is a member of the tumor necrosis factor-receptor family (Montgomery *et al.*, 1996). However, HveA occurs primarily on lymphoid cells so is unlikely to be a major player in HSV entry (Montgomery *et al.*, 1996). The second set of coreceptors belong to the immunoglobulin superfamily. Nectin-1α (also known as HveC or Prr1) and Nectin-2α (also known as HveB or Prr2) were both found to bind gD (Geraghty *et al.*, 1998, Warner *et al.*, 1998). Nectin-1α is found in cell lines of epithelial origin (HEp-2 and Hela) and in brain and spinal cord tissue on human multiple-tissue northern blots (Cocchi *et al.*, 1998), and can mediate entry of wild type HSV-1 (Geraghty *et al.*, 1998). However, Nectin-2α can only mediate entry of HSV-1 mutants but not wild-type virus (Warner *et al.*, 1998). The third family of coreceptors consists of 3-O-sulfated heparan sulfate. 3-O-sulfated heparan sulfates can mediate HSV-1 entry into Chinese hamster ovary cells, but it still has not been shown to mediate entry into human cells (Shukla *et al.*, 1999).

The third step in viral entry is the fusion of the host cell membrane with the virion envelope. The interaction of gD with one of the receptors activates membrane fusion, which requires gB, gH-gL, gD and the receptor (Forrester *et al.*, 1992, Ligas & Johnson, 1988, Sarmiento *et al.*, 1979, Shukla & Spear, 2001). Upon fusion, the nucleocapsid and associated tegument is released into the host cytosol. The fate of the proteins in the tegument is varied: some remain in the cytosol, some are transported to the nucleus and some stay associated with the capsid. The role of the tegument-associated proteins is to create a hospitable environment for viral replication in the hostile host cell. Once inside the host cell the nucleocapsid interacts with the microtubular network (Kristensson *et al.*, 1986, Sodeik *et al.*, 1997) and is transported to the nucleus via the microtubule-motor dynein and its cofactor dynactin (Dohner *et al.*, 2002). The nucleocapsid interacts with the nuclear pore complex (Batterson *et al.*, 1983, Sodeik *et al.*, 1997), which requires importin-β and the interaction triggers the release of the viral DNA into the nucleus (Ojala *et al.*, 2000). Recently, a paper has reported that once released into the nucleus the viral genome remains linear and does not circularize during the course of the lytic infection (Jackson & DeLuca, 2003).

1.5.2 Immediate–Early Gene Expression

Once inside the nucleus, transcription of the linear genome takes place by host RNA polymerase II (RNAP II) (Alwine *et al.*, 1974, Costanzo *et al.*, 1977). HSV genes are transcribed in a coordinately and temporally regulated cascade fashion. HSV genes fall into three kinetic classes : immediate-early (IE or α), early (E or β) and late (L or γ) (reviewed in (Roizman & Knipe, 2001)). The IE genes are transcribed in the absence of *de novo* protein synthesis and stimulate the expression of the E and L genes. The synthesis of IE gene products peaks between 3 and 5 hrs post infection at a multiplicity of infection of 20 (Honess & Roizman, 1974). E proteins peak synthesis between 5 and 7 hrs post infection (Honess & Roizman, 1974) and these proteins are generally

required for DNA replication. The synthesis of L proteins continues at increasing rates until at least 12 hrs after infection (Honess & Roizman, 1974) and L proteins are required for assembly and egress of the virion.

IE gene promoters contain various *cis*-acting elements including, TATA and CAAT elements, binding sites for the transcription factor Sp1 and sometimes binding sites for infected cell protein 4 (ICP4) (Roizman & Knipe, 2001, Weir, 2001). Transcription of IE genes is controlled by inducible enhancer elements that bind multiprotein complexes. The viral regulatory protein VP16 (also called α-TIF or ICP25 or Vmw65), which is encoded by the UL48 gene, is responsible for the induction of IE gene transcription (Batterson & Roizman, 1983, Campbell *et al.*, 1984, Post *et al.*, 1981). The stimulation of transcription by VP16 requires the presence of several copies of a conserved *cis*-acting sequence in the promoter, known as the TAATGART element (where R is a purine residue) (Gaffney *et al.*, 1985, Mackem & Roizman, 1982a, Mackem & Roizman, 1982b). While VP16 interacts with the element only weakly, a multiprotein complex (the VP16-induced complex, VIC) comprising of the ubiquitous transcription factor Oct-1, the host cell factor-1 (HCF, C1 or VCAF) and VP16, interacts with the element with a much higher affinity (Kristie *et al.*, 1989, Kristie & Roizman, 1987, McKnight *et al.*, 1987, O'Hare *et al.*, 1988, O'Hare & Gooding, 1988, Preston *et al.*, 1988). VP16 is a component of the tegument and after delivery into the cytoplasm, HCF-1 directly interacts with VP16 (Gerster & Roeder, 1988, Katan *et al.*, 1990, Kristie & Sharp, 1990, Xiao & Capone, 1990) and delivers it to the nucleus (La Boissiere *et al.*, 1999). In the nucleus, Oct-1 binds to a consensus octamer sequence which overlaps with the enhancer element and VP16 interacts with Oct-1 bound to the viral DNA and with HSV-specific DNA sequences directly adjacent to the octamer motif (Kristie & Sharp, 1990, Pomerantz *et al.*, 1992, Stern & Herr, 1991, Stern *et al.*, 1989). However, the formation of a stable complex requires the participation of HCF-1 (Gerster & Roeder, 1988, Katan *et al.*, 1990, Kristie *et al.*, 1989, Xiao & Capone, 1990). HCF-1 does not bind DNA, but rather interacts with both VP16 and Oct-1

to promote the stable assembly of the VIC (Hayes & O'Hare, 1993, Kristie & Sharp, 1993, Vogel & Kristie, 2000). IE promoters also contain binding sites for other cellular transcription factors, such as, GABP and Sp1 and these factors have been found to significantly contribute to the activation of IE gene transcription (Jones & Tjian, 1985, LaMarco & McKnight, 1989, Triezenberg *et al.*, 1988b). HCF-1 binds to both GABP and Sp1, and it appears that HCF-1 is required for the stable assembly of all enhancer elements into a more complex regulatory unit (Vogel & Kristie, 2000). This may be why HCF-1 is required maximal transactivation of IE gene promoters (Luciano & Wilson, 2002).

VP16 contains two major domains required for its activity. The amino (N)-terminus is required for promoter targeting via interaction with Oct-1and HCF-1 (Kristie & Sharp, 1990, Stern *et al.*, 1989) and binding of DNA (Stern & Herr, 1991). On the other hand, the carboxy (C)-terminus contains an acidic domain required for transcription activation by VP16 (Ace *et al.*, 1988, Greaves & O'Hare, 1989, Triezenberg *et al.*, 1988a, Werstuck & Capone, 1989a) (Werstuck & Capone, 1989b). The VP16 activation domain binds to multiple host transcription factors: TFIID (Ingles *et al.*, 1991, Stringer *et al.*, 1990), TFIIB (Lin & Green, 1991, Lin *et al.*, 1991), TFIIA (Kobayashi *et al.*, 1995), TFIIH (Blau *et al.*, 1996, Xiao *et al.*, 1994), and hTAFII32 (Goodrich *et al.*, 1993, Klemm *et al.*, 1995) as well as the RNAP II holoenzyme (Hengartner *et al.*, 1995). Mutations in VP16 which inhibit binding to these transcription factors, impairs VP16-mediated stimulation of transcription (reviewed in (Flint & Shenk, 1997)). VP16 stimulates the early steps in transcription, such as assembly of preinitiation complexes, initiation, and also elongation, probably by stabilizing the binding of the RNAP II holoenzyme to the promoter (Flint & Shenk, 1997).

There are six IE proteins which are produced following the transactivation of IE gene promoters by VP16. These are ICP0 (RL2), ICP4 (RS1), ICP22 (US1), ICP27 (UL54), ICP47 (US12) and US1.5 (US1.5). All, except ICP47 stimulate E gene expression in at least some types of cells (Roizman & Knipe, 2001).

1.5.3 ICP0

ICP0 is a 110 kDa phosphoprotein that is non-essential for viral replication, but is required for progression through the lytic cycle at low multiplicities of infection (Sacks & Schaffer, 1987, Stow & Stow, 1986). Early transfection studies demonstrated that ICP0 is a promiscuious transactivator and stimulates expression of gene products from viral IE, E and L promoters either by itself or in concert with ICP4 (Cai & Schaffer, 1992, Everett, 1984b, Gelman & Silverstein, 1985, O'Hare & Hayward, 1985a, Quinlan & Knipe, 1985). ICP0 does not require specific promoter sequences (Everett *et al.*, 1991b) and cannot directly bind DNA (Everett *et al.*, 1991a). Thus, ICP0 appears to be affecting transcription indirectly, likely through interactions with other proteins. A number of interactions have been detected which include: a ubiquitin-specific protease (HAUSP or USP7) (Everett *et al.*, 1997), cyclin D3 (Kawaguchi *et al.*, 1997b), elongation factor EF-1δ (Kawaguchi *et al.*, 1997a), transcription factor BMAL-1 (Kawaguchi *et al.*, 2001), the viral transcriptional regulator ICP4 (Yao & Schaffer, 1994), and with RNAP II in a low molecular mass complex (Jenkins & Spencer, 2001). How all of these interactions fit into the role of ICP0 in stimulating the lytic infection remains to be determined.

Early in infection ICP0 is translocated to the nucleus where it localizes to nuclear domain 10 (ND10) structures (Everett & Maul, 1994, Maul & Everett, 1994, Maul *et al.*, 1993), which are believed to play a role in the host anti-viral repression system. ICP0 causes the disruption of the ND10 sites (Maul *et al.*, 1993) (Everett & Maul, 1994, Maul & Everett, 1994), and is then translocated to the cytoplasm (Kawaguchi *et al.*, 1997b, Lopez *et al.*, 2001). This disruption requires the ring finger domain of ICP0 (Maul & Everett, 1994, Maul *et al.*, 1993) and is proteasome dependent (Chelbi-Alix & de The, 1999, Everett *et al.*, 1998a, Muller & Dejean, 1999). ND10 contain two major components, promyelocytic leukemia protein (PML) and Sp100, both of which are conjugated to the ubiquitin-like protein SUMO-1. ICP0 causes the proteasome-dependent

degradation of the PML and Sp100 isoforms conjugated to SUMO-1 (Chelbi-Alix & de The, 1999, Everett *et al.*, 1998a, Muller & Dejean, 1999, Parkinson & Everett, 2000). This is possibly through HAUSP (Everett *et al.*, 1999b, Everett *et al.*, 1997) and/or a SUMO-1-specific protease SENP-1 (Bailey & O'Hare, 2002) both of which may cleave SUMO-1 from PML or Sp100 and allow for their ubiquitination and subsequent degradation. The degradation of PML and Sp100 correlates with the disruption of ND10 (Muller & Dejean, 1999, Parkinson & Everett, 2000), which is not surprising as PML is required for the stable assembly of ND10 (Ishov *et al.*, 1999).

ICP0 also causes the proteasome dependent degradation of the catalytic subunit of DNA dependent protein kinase (Parkinson *et al.*, 1999) and centromere proteins CENP-A (Lomonte *et al.*, 2001) and CENP-C (Everett *et al.*, 1999a). The proteasome inhibitor MG132 blocks the ability of ICP0 to stimulate viral infection and reactivation from quiescence (Everett *et al.*, 1998b). As well, the ring finger domain found in its N-terminus has been found in other E3 ligases to mediate the transfer of ubiquitin to targeted substrates (Joazeiro & Weissman, 2000). These observations suggested that ICP0 functions via the ubiquitin-proteasome pathway. Indeed, ICP0 has been found to induce the formation of colocalizing conjugated ubiquitin in infected and transfected cells (Everett, 2000). In addition, ICP0 was found to act as an E3 ubiquitin ligase and stimulates the synthesis of conjugated ubiquitin *in vitro* in the presence of E1 and E2 enzymes, UbcH5a and UbcH6 (Boutell *et al.*, 2002), and the E2 cdc34 enzyme (Van Sant *et al.*, 2001). Thus, the role of ICP0 in the lytic cycle is linked to the ubiquitin-proteasome system by affecting the stability of cellular proteins and nuclear structures.

ICP0 has also been found to play a role in inhibiting the interferon (IFN) and antiviral responses induced by the virion very early on during infection. HSV-1 has been found to induce both IFN-dependent and IFN-independent antiviral states, and inhibits both responses. The IFN-independent antiviral response is characterized by the induction of IFN-stimulated genes without the

need for IFN (Mossman *et al.*, 2001, Nicholl *et al.*, 2000). HSV-1 appears to encode multiple gene products able to inhibit this response (Mossman *et al.*, 2001), and one such gene product is ICP0 (Eidson *et al.*, 2002). ICP0 inhibits the induction of IFN-stimulated genes in a proteasome-dependent manner (Eidson *et al.*, 2002). The IFN response pathway is also affected by ICP0. ICP0 is required to overcome the antiviral repression mediated by IFN-α (Harle *et al.*, 2002, Mossman & Smiley, 2002) and β (Harle *et al.*, 2002). ICP0 overcomes a distinct and possibly earlier IFN-induced pathway as compared to ICP34.5, which counteracts the protein kinase PKR pathway (Mossman & Smiley, 2002). The expression of ND10 proteins PML and Sp100 is increased in response to IFN (Grotzinger *et al.*, 1996, Guldner *et al.*, 1992, Lavau *et al.*, 1995) and the PML promoter contains IFN response elements (Stadler *et al.*, 1995). Recently, it was found that PML, in part, mediates the antiviral state induced by IFN and it appears that ICP0 targets PML (Chee *et al.*, 2003) and consequently ND10 for destruction to preclude the development of an antiviral response by IFN. Thus, ICP0 appears to promote the lytic cycle by inhibiting antiviral repression mechanisms of the host cell.

1.5.4 ICP4

ICP4 is a 175 kDa phosphoprotein which is encoded by the RS1 gene which is present in two copies in the HSV genome. ICP4 is essential for viral replication as it is required for transcriptional activation of most E and L genes (DeLuca & Schaffer, 1987, Everett, 1984b, Gelman & Silverstein, 1985, Godowski & Knipe, 1986). In the absence of ICP4, the E genes are expressed poorly and the other IE genes are overexpressed compared to the wild type virus (DeLuca *et al.*, 1985, Dixon & Schaffer, 1980, Preston, 1979). However, the overexpression of the IE genes partly reflects the lack of ICP4's ability to repress transcription of certain genes (DeLuca & Schaffer, 1985, Godowski & Knipe, 1986, Gu *et al.*, 1993, O'Hare & Hayward, 1985b). ICP4 binds DNA as a homodimer (Metzler & Wilcox, 1985) and localizes to the nucleus of infected

cells (Courtney & Benyesh-Melnick, 1974). Early in infection ICP4 forms discrete foci in the nucleus, some of which are associated with ND10 (Everett *et al.*, 2003). Some of the ICP4 foci develop into viral replication compartments, suggesting that these foci may represent ICP4 molecules being recruited onto parental viral genomes (Everett *et al.*, 2003). Indeed, it appears that ICP4 is one component necessary to localize the viral genome to ND10 (Tang *et al.*, 2003).

ICP4 acts as a repressor of transcription on its own gene, *ORF P*, *ORF O*, and LAT (DeLuca & Schaffer, 1987, O'Hare & Hayward, 1985b, Rivera-Gonzalez *et al.*, 1994, Yeh & Schaffer, 1993). These genes contain the strongest affinity binding sites for ICP4 spanning the transcription initiation sites (Faber & Wilcox, 1986, Kristie & Roizman, 1984, Muller, 1987). The strongest binding sites for ICP4 are known to repress rather than activate transcription, and the effectiveness of repression depends on the distance of the binding site from the transcription initiation site (Roizman & Knipe, 2001). The DNA binding activity of ICP4 is essential for its action as a repressor of transcription (Gu *et al.*, 1993, Roberts *et al.*, 1988), however, repression also appears to involve specific ICP4 interactions with the transcription factors, TATA-binding protein (TBP), and TFIIB (Gu *et al.*, 1995, Kuddus *et al.*, 1995).

As mentioned above, ICP4 activates transcription from E and L promoters, however, the exact mechanism by which this is achieved is not fully understood. The DNA-binding domain is essential for transactivation (Paterson & Everett, 1988b, Shepard *et al.*, 1989), however a consensus sequence that is strongly bound by ICP4 and present in all promotors activated by ICP4 has not yet been found (Everett, 1987, Wagner *et al.*, 1995). The minimal *cis*-acting elements required for transactivation by ICP4 are the presence of a TATA-box and a eukaryotic initiator sequence (Inr) (Cook *et al.*, 1995, Everett, 1984a, Homa *et al.*, 1988, Johnson & Everett, 1986, Kim *et al.*, 2002). The high levels of L gene transcription are probably due in large part to ICP4 functioning through the Inr element (Kim *et al.*, 2002). It is likely that ICP4 activates transcription through interactions with cellular transcription factors. ICP4

interacts with TBP (Gu *et al.*, 1995, Smith *et al.*, 1993), the TBP-containing general transcription factor TFIID (Gu & DeLuca, 1994) through its TBP-associated factor (TAF) 250 subunit (Carrozza & DeLuca, 1996), TFIIB (Smith *et al.*, 1993) (Gu *et al.*, 1995) and the cellular high mobility group factor 1 (HMG1) (Carrozza & DeLuca, 1998). HMG1 may aid the ICP4 interaction with TFIID by its ability to bend DNA (Carrozza & DeLuca, 1998). ICP4 can form tripartite complexes with TBP, TFIIB and TFIID on DNA which contains a TATA box and a ICP4 binding site in the proper position (Kuddus *et al.*, 1995, Smith *et al.*, 1993). ICP4 also enhances the binding of TFIID to the TATA box which promotes the formation of transcription preinitiation complexes on promoters (Grondin & DeLuca, 2000). Thus, it appears that ICP4 functions at the earliest stages of transcription.

ICP4 mutants which consist of the N-terminal ~ 800 amino acids can activate transcription *in vitro* (Carrozza & DeLuca, 1996) and during virus infection (DeLuca & Schaffer, 1988). These mutants can also activate transcription of many β and βγ genes (DeLuca & Schaffer, 1988), repress transcription from promoters (DeLuca & Schaffer, 1988, Gu *et al.*, 1995), form tripartite complexes with TFIID and TBP on promoters (Smith *et al.*, 1993), and can promote the formation of preinitiation complexes (Grondin & DeLuca, 2000). Residues in the N-terminal 796 amino acids also include a transactivation domain (Xiao *et al.*, 1997), contribute to regulation of ICP4 functions by ICP27 (Samaniego *et al.*, 1995), are required for homodimerization (Gallinari *et al.*, 1994), DNA-binding (Everett *et al.*, 1990, Wu & Wilcox, 1990) and contains a nuclear localization signal (NLS) (Paterson & Everett, 1988a). However, mutants lacking the C-terminal region of ICP4 are defective for full activation of E genes and DNA synthesis, and L genes are not expressed (DeLuca & Schaffer, 1988). Consequently, these viruses are severely impaired for replication. Thus, the C-terminal region of ICP4 is required for full-transactivation activity and viral growth. However, it appears that it does not function as an independent transactivation domain, but rather as an enhancer of

the ICP4 N-terminal transactivation domain (Bruce & Wilcox, 2002). Thus, full activation of transcription most likely requires motifs in both the N- and C-termini (Bruce & Wilcox, 2002). The C-terminus of ICP4 also includes domains which interact with TAFII250 (Carrozza & DeLuca, 1996), ICP0 (Yao & Schaffer, 1994), and ICP27 (Panagiotidis *et al.*, 1997).

1.5.5 ICP22/Us1.5

ICP22 has an apparent size of 68 kDa, and is one of two products of the α22 gene. Us1.5 protein is the other product, and the ORF is identical to the middle and C-terminal of ICP22 (Carter & Roizman, 1996). Both are expressed as IE proteins, and are considered non-essential for viral replication in tissue culture (Ogle & Roizman, 1999, Poffenberger *et al.*, 1993, Post & Roizman, 1981). However, it appears that ICP22 does show some cell-type dependent activity, as it is not required in Hep-2 or Vero cells, but is required for optimal virus replication in primary human cells and in rabbit and rodent cells (Poffenberger *et al.*, 1993, Purves *et al.*, 1993, Sears *et al.*, 1985). ICP22 is a nuclear protein which localizes in small dense nuclear bodies early in infection (Jahedi *et al.*, 1999). After the onset of DNA synthesis, ICP22 localizes to defined transcriptional complexes associated with L gene expression which also contain ICP4, RNAP II, a host nucleolar protein, and viral DNA (Leopardi *et al.*, 1997). The association of ICP22 with these structures is dependent on UL13 and US3, the viral protein kinases (Leopardi *et al.*, 1997). Later in infection, ICP22 relocalizes to small nuclear bodies (Markovitz & Roizman, 2000). Thus, ICP22 may exert different effects at early and late times of infection at distinct nuclear sites.

ICP22 plays a role in the regulation of HSV gene expression. ICP22 is required for the efficient expression of ICP0 (Purves *et al.*, 1993), and influences the splicing pattern of α0 mRNA (Carter & Roizman, 1996). ICP22 is also required for the efficient expression of a subset of L genes (γ2) exemplified by the products of the US11, UL38 and UL41 genes (Purves *et al.*, 1993, Sears *et*

al., 1985). In addition to ICP22, UL13 is also required for the expression of this subset of L proteins (Purves *et al.*, 1993). The role of ICP22 is intimately tied to the viral protein kinase UL13, as viral mutants defective for UL13 exhibit a similar phenotype as ICP22 mutants (Purves *et al.*, 1993, Purves & Roizman, 1992). ICP22 is extensively posttranslationally modified and this appears to modulate its activity. These modifications include, phosphorylation (Purves *et al.*, 1993, Purves & Roizman, 1992), nucleatidylation by casein kinase II (Mitchell *et al.*, 1997), and tyrosine phosphoryation (Blaho *et al.*, 1997, O'Toole *et al.*, 2003). Both UL13 and US3 phosphorylate ICP22 and US1.5 (Purves *et al.*, 1993, Purves & Roizman, 1992).

HSV promotes the transcription of its genes by altering the host RNAP II (Jenkins & Spencer, 2001, Long *et al.*, 1999, Rice *et al.*, 1995, Rice *et al.*, 1994). The largest subunit of RNAP II contains the catalytic activity and posses a C-terminal structure called the C-terminal domain (CTD). The CTD is essential for cell viability and is extensively phosphorylated (reviewed in (Dahmus, 1996, Emili & Ingles, 1995)). RNAP II is normally present in two forms, RNAP IIO (hyperphosphorylated), and RNAP IIA (hypophosphorylated). RNAP IIA is involved in transcription initiation and RNAP IIO is involved in transcription elongation (Lu *et al.*, 1991, Payne *et al.*, 1989). During HSV infection, there is a depletion of RNAP IIO and RNAP IIA and a new intermediately phosphorylated transcriptionally active form of RNAP II appears, RNAP IIi (Rice *et al.*, 1994). ICP22 and UL13 mutants are deficient for the induction of RNAP IIi, the depletion of RNAP IIA, and effective viral transcription does not occur at late times of infection (Long *et al.*, 1999, Rice *et al.*, 1995). Thus, ICP22 and UL13 appear to be required to alter the phosphorylation of the CTD of RNAP II and subsequently promote late gene transcription on certain cell lines.

ICP22 has recently been found to interact functionally with the cyclin-dependent kinase cdc2 (cdk1), which is required for the optimal expression of the same subset of $\gamma 2$ proteins (exemplified by US11) which also requires ICP22 and UL13 (Advani *et al.*, 2000a, Advani *et al.*, 2000b, Advani *et al.*,

2003). During HSV infection cdc2 is posttranscriptionally modified, stabilized, and activated, while surprisingly its natural partners cyclinA and cyclin B are degraded (Advani *et al.*, 2000a). The posttranslational modification and activation of cdc2 and the degradation of the cyclins requires the presence of ICP22 and UL13 (Advani *et al.*, 2000a). Cdc2 interacts with the viral DNA polymerase processivity factor, UL42 (Advani *et al.*, 2001), and in turn the cdc2-UL42 complex recruits a phosphorylated form of topoisomerase IIα (Advani *et al.*, 2003). This complex has been suggested to mediate the expression of the subset of γ2 genes exemplified by US11 (Advani *et al.*, 2003). ICP22 mediates the posttranslational modification of topoisomerase IIα, which enables the interaction of cdc2-UL42 with topoisomerase IIα (Advani *et al.*, 2003). These data suggest that ICP22 mediates the expression of a subset of γ2 genes by inducing the formation of the cdc2-UL42-topoisomerase IIα complex.

1.5.6 ICP27

ICP27 is a 63 kDa protein which is absolutely essential for viral replication in tissue culture (McCarthy *et al.*, 1989, Rice & Knipe, 1990, Sacks *et al.*, 1985). ICP27 carries out multiple regulatory functions that are indispensable during the viral lytic infection. First, ICP27 is required for the efficient expression of several E and most L gene mRNAs and proteins (McCarthy *et al.*, 1989, McGregor *et al.*, 1996, Rice & Knipe, 1990, Rice *et al.*, 1993, Sacks *et al.*, 1985, Uprichard & Knipe, 1996). Second, ICP27 stimulates viral DNA replication by approximately 10-fold (McCarthy *et al.*, 1989, Rice & Knipe, 1990). This effect is most likely due to ICP27's ability to promote the expression of E gene products required for DNA replication (McGregor *et al.*, 1996, Uprichard & Knipe, 1996). However, ICP27 stimulates the transcription of L genes independently of stimulating E gene expression and viral DNA replication (Jean *et al.*, 2001, Rice & Knipe, 1990). Third, ICP27 represses several E and L genes at late times after infection (McCarthy *et al.*, 1989, Rice & Knipe, 1990, Sacks *et al.*, 1985). Fourth, ICP27 represses the production of host mRNAs and proteins during

infection which contributes to the shutoff of host gene expression (Hardwicke & Sandri-Goldin, 1994, Sacks *et al.*, 1985, Soliman *et al.*, 1997, Spencer *et al.*, 1997). Fifth, ICP27 contributes to the prevention of virus-induced apoptosis during infection (Aubert & Blaho, 1999, Goodkin *et al.*, 2003). It is required for the activation and translocation of NF-κB to the nucleus in virally infected cells to prevent apoptosis (Goodkin *et al.*, 2003). Sixth, ICP27 is required for blocking the cell cycle in the G1 phase during infection (Song *et al.*, 2001).

At least some of ICP27's effects appear to be mediated through transcriptional modulation. ICP27 promotes the transcription of at least two L genes, UL44 and UL47 (Jean *et al.*, 2001), and inhibits host gene transcription (Spencer *et al.*, 1997). ICP27 interacts with both ICP0 and ICP4 (Mullen *et al.*, 1995, Panagiotidis *et al.*, 1997), and alters the phosphorylation and DNA-binding ability of ICP4 (Panagiotidis *et al.*, 1997, Xia *et al.*, 1996). Both ICP4 and ICP27 colocalize to replication compartments (de Bruyn Kops *et al.*, 1998) and are required to localize transcriptionally active viral DNA to ND10 (Tang *et al.*, 2003). In addition, ICP27 interacts with RNAP II (Jenkins & Spencer, 2001, Zhou & Knipe, 2002), and is required for the association of ICP8 with RNAP II (Zhou & Knipe, 2002). This interaction may reflect ICP27's role in stimulating E and L gene expression and/or its role in inhibiting host transcription.

The molecular mechanisms by which ICP27 mediates its various functions are still poorly understood. However, it appears that ICP27 alters almost every aspect of mRNA metabolism, including transcription, polyadenylation, splicing, and nuclear export. ICP27 exerts at least some of its effects at the post transcriptional level. It can alter the specificity of pre-mRNA polyadenylation machinery by promoting the use of weak (non-consensus) polyadenylation sites (McGregor *et al.*, 1996), and downstream polyadenylation sites (Hann *et al.*, 1998). Thus, ICP27 may stimulate the expression of L genes containing inherently weak polyadenylation sites (McGregor *et al.*, 1996). ICP27 also inhibits pre-mRNA splicing and functions as a transport factor for the nuclear export of intronless mRNAs (see below for more details).

ICP27 is required for the efficient shutoff of cellular protein synthesis (Hardwicke & Sandri-Goldin, 1994, McCarthy *et al.*, 1989, Sacks *et al.*, 1985, Soliman *et al.*, 1997) and is thought to contribute to the shutoff process by inhibiting pre-mRNA splicing (Hardwicke & Sandri-Goldin, 1994, Hardy & Sandri-Goldin, 1994, Soliman *et al.*, 1997). Therefore the maturation of intron-containing transcripts would be stopped and they would be prevented from reaching the cytoplasm. A significant amount of data has supported the notion that ICP27 impairs pre-mRNA splicing. For instance, nuclear extracts from infected cells have a lower splicing activity and this defect requires the presence of ICP27 (Hardy & Sandri-Goldin, 1994). ICP27 co-immunoprecipitates with various splicing factors and altered the phosphorylation state of at least two proteins (Sandri-Goldin & Hibbard, 1996). ICP27 colocalizes with and redistributes splicing factors in the nuclei of infected cells (Phelan *et al.*, 1993, Sandri-Goldin & Hibbard, 1996, Sandri-Goldin *et al.*, 1995). ICP27 interacts with the essential splicing factor, splicesome-associated protein145 (SAP145) (Bryant *et al.*, 2001). More recent evidence has found that ICP27 blocks splicesome assembly early on at the pre-splicesomal complex A stage (Bryant *et al.*, 2001, Lindberg & Kreivi, 2002, Sciabica *et al.*, 2003). Additionally, ICP27 has been shown to interact with SR protein kinase 1 (SRPK1) and alter its activity to result in the hypophosphorylation of SR proteins (splicing factors), thus impairing their ability to function in splicesome assembly (Sciabica *et al.*, 2003). The inhibition of splicing by ICP27 is a temporary and reversible phenomenon that takes place at early times during infection (Sciabica *et al.*, 2003). However, splicing is not completely blocked in HSV-infected cells, as ICP27 does not alter the rate of accumulation of spliced α-globin mRNA in infected cells (Ellison *et al.*, 2000) (Cheung *et al.*, 2000) and does not inhibit splicing of cellular transcripts in *Xenopus* oocytes (Koffa *et al.*, 2001).

In the eukaryotic cell, the processes of transcription, polyadenylation, splicing and mRNA export are linked. However, the majority of HSV transcripts are intronless and therefore require a way to access cellular export pathways

efficiently. ICP27 mediates the export of intronless viral mRNAs (Chen *et al.*, 2002, Cheung *et al.*, 2000, Koffa *et al.*, 2001, Sandri-Goldin, 1998, Soliman *et al.*, 1997). It binds RNA through its arginine-rich RGG box (Mears & Rice, 1996, Sandri-Goldin, 1998) and shuttles between the nucleus and cytoplasm (Sandri-Goldin, 1998, Soliman *et al.*, 1997) (Mears & Rice, 1998, Phelan & Clements, 1997). ICP27 also contains two nuclear localization signals (NLS) (Mears *et al.*, 1995) and a leucine-rich nuclear export sequence (NES) (Sandri-Goldin, 1998). The deletion of the NES restricts ICP27 to the nucleus and impairs the viability of the virus (Lengyel *et al.*, 2002). ICP27 was originally thought to export mRNAs through the cellular export adapter CRM1 (Soliman & Silverstein, 2000). However, CRM1 is not required for mRNA export by ICP27 in *Xenopus* oocytes (Koffa *et al.*, 2001) and the NES does not require CRM1 for its export activity (Chen *et al.*, 2002). More recently, ICP27 has been found to bind the cellular export factors Aly/REF and TAP (Chen *et al.*, 2002, Koffa *et al.*, 2001). The current model for ICP27–mediated export is that ICP27 recruits Aly/REF and TAP to viral intronless mRNAs as a way of accessing the cellular TAP mRNA export pathway (Chen *et al.*, 2002, Koffa *et al.*, 2001). Interestingly, the deletion of the Aly/REF interaction domain (Koffa *et al.*, 2001) has very little effect on virus growth and gene expression in Vero cells (Lengyel *et al.*, 2002). Thus, the ICP27-Aly/REF interaction may be dispensable, at least in some cell types.

1.5.7 ICP47

ICP47 is a 12 kDa protein and is the only IE gene which does not play a role in the regulation of viral gene expression. ICP47 is not essential in cell culture (Longnecker & Roizman, 1986) but rather plays a role in immune evasion in the host. It binds to the transporter associated with antigen processing (Fruh *et al.*, 1995, Hill *et al.*, 1995) and prevents the translocation of peptides into the endoplasmic reticulum (Fruh *et al.*, 1995, Hill *et al.*, 1995, York *et al.*, 1994). Therefore, assembly of class I major histocompatibility complex (MHC) heavy chains with antigenic peptides does not occur and empty MHC

class I molecules are retained in the endoplasmic reticulum (Fruh *et al.*, 1995, Hill *et al.*, 1995, York *et al.*, 1994). ICP47, along with vhs, effectively renders the host cell resistant to lysis by HSV-specific CD8+ cytotoxic T lymphocytes (CTLs) by preventing antigen presentation by MHC class I molecules on the surface of the cell (Fruh *et al.*, 1995, Hill *et al.*, 1995, Tigges *et al.*, 1996). Recently, ICP47's ability to evade CTLs has been used in vectors for xenotransplantation (Crew & Phanavanh, 2003), gene therapy (Scaria *et al.*, 2000) and transplantation to prevent rejection of tissue grafts and prolong transgene expression (Furukawa *et al.*, 2000).

1.5.8 Early Gene Expression and Viral DNA Replication

The next set of HSV genes which are expressed are the E (or β) genes. The E genes are genes that require at least the presence of functional ICP4 but not viral DNA replication to be transcribed (Honess & Roizman, 1974, Honess & Roizman, 1975). E protein synthesis peaks at about 5 to 7 hrs after infection at a multiplicity of infection of 20 (Honess & Roizman, 1974). These proteins are involved in DNA synthesis and nucleic acid metabolism. Thus, the appearance of the E proteins signals the onset of viral DNA replication, which in turn promotes the expression of the L genes (Roizman & Knipe, 2001). The E genes can be divided into two general groups (Roizman & Knipe, 2001). The first is the β1 genes, which are expressed very early after infection or almost concurrently with IE gene expression. This group is exemplified by two main genes: UL29, which encodes ICP8, the single-stranded DNA binding protein; and UL39, which encodes, ICP6 the large subunit of nucleotide reductase. The second group is the β2 genes, which are more heterogeneous with respect to their peak polypeptide synthesis times, but generally more time after the onset of IE gene expression takes place before their expression. These genes are exemplified by UL23, which encodes thymidine kinase and UL30, which encodes the HSV polymerase. Additionally, ICP27 is required for the expression of some of these genes (Uprichard & Knipe, 1996). E gene promoters contain binding sites for a

variety of cellular transcription factors upstream of an essential TATA element (Roizman & Knipe, 2001). However, the composition and arrangement of these binding sites differs considerably in E gene promoters, suggesting that the organization and context of these elements determine the expression of the gene.

After E gene expression begins, a number of the E proteins localize to prereplicative sites near ND10 where they assemble onto the viral genome (Ishov & Maul, 1996). Later, during viral replication, globular structures called replication compartments form where DNA synthesis proceeds in earnest (Quinlan *et al.*, 1984). Seven viral gene products are necessary and sufficient to replicate the HSV genome in cell culture (Challberg, 1986, Wu *et al.*, 1988). These are: UL30, the viral DNA polymerase; UL42, the processivity factor and together with UL30 forms the HSV DNA polymerase holoenzyme; UL29, the ICP8 ssDNA-binding protein; UL9, the origin-binding protein; and UL5, UL8 and UL52, which form the helicase-primase. The contribution of host factors in HSV DNA synthesis has not yet been determined, although they are required. The HSV genome contains three origins of replication (Roizman & Knipe, 2001). The first and second is *ori S*, which is located in the internal repeats of the short region, and so is present in two copies. The third is *ori L*, which is located in the unique long region between the UL29 and UL30 genes. The reason for three origins of replication is not yet clear at this time, but neither origin is essential for viral replication (Igarashi *et al.*, 1993, Polvino-Bodnar *et al.*, 1987).

The prevailing model of viral DNA synthesis for many years was that a circular HSV genome was initially replicated by a theta replication mode and then switched to a rolling-circle mechanism of replication (Roizman & Knipe, 2001). However, recently it has been found that the HSV genome does not circularize during the lytic infection (Jackson & DeLuca, 2003). Circular genomes could not be detected either in the presence or absence of viral DNA replication and ICP0 was found to inhibit the formation of circular genomes (Jackson & DeLuca, 2003). Thus, it is likely that circular genomes are not the

initial replication template. The current model is that genome replication may proceed from the three origins on linear genomes to produce highly branched structures that may be modified and/or resolved by recombination or by the viral packaging machinery (Jackson & DeLuca, 2003). Thus, this model of HSV replication will have to be further developed and tested in future experiments.

1.5.9 Late Gene Expression, Virion Assembly and Egress

The L genes are expressed at peak times after viral DNA synthesis begins, and their expression level is enhanced by viral DNA replication (Roizman & Knipe, 2001). The transcription of L genes takes place in replication compartments (Phelan *et al.*, 1997) and protein synthesis peaks between 7 and 9 hrs after infection (Honess & Roizman, 1974, Honess & Roizman, 1975). The L genes generally encode virion structural proteins. There are two main groups of L genes, the first are the early-late or γ1 and the second are the true-late or γ2 genes (Roizman & Knipe, 2001). The γ1 genes are genes which are expressed early and whose expression is enhanced by viral DNA replication. These genes include: UL19 (ICP5), UL27 (gB), and US6 (gD). The γ2 genes require viral DNA synthesis for any appreciable expression of their gene products. These genes include: UL44 (gC), US11, US9, and UL49.5. The γ1 promoters generally contain one or two binding sites for cellular transcription factors upstream from the TATA element as well as an initiator element at transcription initiation site (Huang & Wagner, 1994, Lieu & Wagner, 2000). The γ2 promoters do not have any *cis*-acting regulatory elements upstream from their TATA box, but do have a downstream activator element (Homa *et al.*, 1986, Kibler *et al.*, 1991, Mavromara-Nazos & Roizman, 1989). Late gene expression also requires many of the IE proteins. ICP0, ICP4 and ICP27 are all needed for efficient L gene expression and ICP22 is also needed for the expression of a subset of γ2 genes (Roizman & Knipe, 2001). The E protein ICP8 is also needed to simulate L gene expression (Chen & Knipe, 1996, Gao & Knipe, 1991). However, while these viral proteins are needed for gene

expression, the L promoters do not contain specific binding sites for these proteins (Smiley *et al.*, 1992).

Once the L capsid proteins are synthesized, they localize to the cell nucleus and the assembly of capsids takes place in structures called assemblons (Nalwanga *et al.*, 1996) or in replication compartments (de Bruyn Kops *et al.*, 1998, Lamberti & Weller, 1998). The assembly of capsids requires six L gene proteins: VP5,VP19C, VP21, VP22a, VP23 and VP24 and VP26 (Tatman *et al.*, 1994). The formation of the capsids begins with VP21, pre-VP22a and VP24 forming a scaffold structure around which the shell proteins form the capsid (Homa & Brown, 1997, Roizman & Knipe, 2001). Pre-VP22a is the major scaffolding protein, but VP21 can also serve as one. The UL26 protein product cleaves autoproteolytically to yield VP21 and VP24, which is also a protease (Preston *et al.*, 1994). VP24 or UL26 is also required to cleave pre-VP22a into VP22a in the maturation step. There are four major proteins which form the capsid around the scaffold structure (Homa & Brown, 1997, Roizman & Knipe, 2001). These are: VP5, which is the major capsid protein and is the structural subunit of all 162 capsomeres; VP19C and VP23, which are located in the spaces between the capsomers; and VP26, which forms the outer tip of the hexons. During encapsidation of DNA, the scaffolding proteins and the protease are removed from the core of the capsid.

The encapsidation of viral DNA takes place in replication compartments (Lamberti & Weller, 1998) and involves the cleaving of branched genome structures into single genome lengths and the packaging of the linear genomes into a capsid (reviewed in (Homa & Brown, 1997)). The branched DNA is cleaved into unit-length genomes at two *cis*-acting DNA packaging elements, *pac1* and *pac2* (Deiss *et al.*, 1986, Smiley *et al.*, 1990, Varmuza & Smiley, 1985). There are six viral genes which are essential for this process: UL6, UL15, UL17, UL28, UL32 and UL33 (Roizman & Knipe, 2001). UL15, UL28 and UL33 interact in a complex and this complex may function as the viral terminase which

binds both the viral DNA and capsid and cleaves the DNA (Beard *et al.*, 2002). The precise function of the other proteins remains to be defined.

The current model for acquisition of the viral envelope is through envelopment, deenvelopment and reenvelopment of the HSV capsid (reviewed in (Farnsworth *et al.*, 2003, Mettenleiter, 2002)). The capsid acquires a primary lipid envelope by budding through the inner nuclear membrane into the preinuclear space (Farnsworth *et al.*, 2003, Mettenleiter, 2002). Several viral proteins have been implicated in this process, including UL34, UL31 and UL11 (Baines & Roizman, 1992, Reynolds *et al.*, 2001, Roller *et al.*, 2000). UL34 and UL31 form a complex that localizes to the nuclear membrane and is essential for optimal primary envelopment of nucleocapsids (Reynolds *et al.*, 2001, Roller *et al.*, 2000). UL34 also interacts with VP5, the major capsid protein, suggesting that UL34 may direct binding of the capsid to the nuclear membrane (Ye *et al.*, 2000). UL11 has been implicated in the formation of free enveloped virions in the perinuclear space (Loomis *et al.*, 2001). The viral kinase, US3 plays a role in the deenvelopment of nucleocapsids during fusion of the virion envelope with the outer nuclear membrane (Reynolds *et al.*, 2002). In the cytoplasm the nucleocapsids acquire their tegument (Mettenleiter, 2002). The first layer of tegument appears to be icosahedrally structured and composed of UL36 protein which interacts with VP5 (McNabb & Courtney, 1992, Mettenleiter, 2002). The second layer is proposed to be composed of UL37 protein which interacts with UL36 (Mettenleiter, 2002). The subsequent steps in tegumentation are still largely undefined. Other tegument proteins include: VP16, VP22, ICP0, ICP4, US3, US11 and vhs. The tegument-coated capsids then bind onto cytoplasmic membranes, including the *trans*-Golgi network and endosomes (Farnsworth *et al.*, 2003, Johnson & Huber, 2002). These membranes are enriched in viral glycoproteins that bridge the virion envelope onto nucleocapsids through binding tegument proteins. So far two tegument proteins have been implicated in this process. VP22 has been found to interact with gE and gM (Fuchs *et al.*, 2002) and VP16 interacts with gH and gD (Farnsworth *et al.*, 2003, Johnson *et*

al., 1984). The virions bud into the lumen of the cytoplasmic vesicles and acquire their envelope. The HSV glycoproteins gD and gE/gL are redundant and act to anchor the envelope onto tegument-coated capsids (Farnsworth *et al.*, 2003). Either one is required for efficient envelopment (Farnsworth *et al.*, 2003). The enveloped virions are then transported to the cell surface where they are delivered into the extracellular environment.

1.6 Virion Host Shutoff

HSV employs a number of strategies to allow for efficient viral gene expression, one of which is the shutoff of host protein synthesis. The shutoff of host protein synthesis takes place in two phases. The first, termed "early shutoff", occurs early in infection and is mediated by a virion-associated protein and as such, does not require *de novo* viral gene expression. The second phase is called "delayed shutoff", and occurs later in infection and requires *de novo* viral protein synthesis. This distinction between the two phases of shutoff came from the analysis of a HSV-1(HFEM) temperature sensitive mutant *ts*B7. The mutant *ts*B7 can grow and inhibit host protein synthesis at the permissive temperature (34°C), but cannot do either at the non-permissive temperature (39°C) (Fenwick & Clark, 1982, Knipe *et al.*, 1981). Ultraviolet (UV) inactivated *ts*B7 (which cannot express any viral genes) can still suppress synthesis of cellular proteins at 34°C, showing that the early shutoff function does not requires *de novo* viral gene expression (Fenwick & Clark, 1982). A temperature-resistant revertant virus of *ts*B7, RC2, was isolated and could now grow at 39°C and induce delayed shutoff of host protein synthesis at late times post-infection, but it could not induce early shutoff (Fenwick & Clark, 1982). These results indicated that delayed shutoff required *de novo* viral protein synthesis and early and delayed shutoff can occur independently of each other (Fenwick & Clark, 1982). These two phases were confirmed with the isolation and characterization of a number of viral mutants which were unable to induce early shutoff but were capable of inducing delayed shutoff of host protein synthesis (Read & N., 1983).

As mentioned in the ICP27 section, ICP27 is thought to be the viral protein responsible for mediating delayed shutoff through its effects on RNA metabolism, particularly inhibition of splicing.

Early shutoff during HSV infection is characterized by the shutoff of host protein synthesis, the disruption of pre-formed polyribosomes, and the degradation of cellular mRNAs at early times of infection (Fenwick & Clark, 1982, Fenwick & Everett, 1990a, Fenwick & Everett, 1990b, Fenwick & McMenamin, 1984, Fenwick & Owen, 1988, Fenwick & Walker, 1978, Jones *et al.*, 1995, Nishioka & Silverstein, 1977, Nishioka & Silverstein, 1978, Oroskar & Read, 1987, Oroskar & Read, 1989, Read *et al.*, 1993, Read & N., 1983, Roizman *et al.*, 1965, Schek & Bachenheimer, 1985, Smibert & Smiley, 1990, Sydiskis & Roizman, 1966, Sydiskis & Roizman, 1967) (Sydiskis & Roizman, 1968). Studies using virion-associated host shutoff (*vhs*) mutants, in particular the *vhs*1 mutant, showed that these mutants lacked the ability to shutoff host protein synthesis at early times, disrupt polyribosomes, and destabilize mRNAs, suggesting that all these processes are mediated by the same virion-associated component (Fenwick & Everett, 1990b, Kwong & Frenkel, 1987, Kwong *et al.*, 1988, Oroskar & Read, 1989, Strom & Frenkel, 1987). However, it is still unclear if host protein shutoff occurs solely as a consequence of mRNA degradation. One study found that the inhibition of protein synthesis was much greater than the amount of mRNA degradation, suggesting that an additional function was required for full inhibition of host protein synthesis (Schek & Bachenheimer, 1985). Additionally, the dissociation of polyribosomes before mRNA degradation occurred has been observed (Nishioka & Silverstein, 1978).

The destabilization of cellular mRNAs is thought to aid viral mRNAs in accessing the cellular translation machinery, by alleviating competition from cellular mRNAs. Additionally, the *vhs* function also destabilizes viral mRNAs belonging to all kinetic classes (Kwong & Frenkel, 1987, Kwong *et al.*, 1988, Oroskar & Read, 1987, Oroskar & Read, 1989, Strom & Frenkel, 1987). During *vhs*1 infection, the half-lives of viral mRNAs are longer than in wild-type

infections (Kwong & Frenkel, 1987, Oroskar & Read, 1987, Strom & Frenkel, 1987), viral IE and E transcripts accumulate until late times, and L gene expression is delayed and reduced (Kwong & Frenkel, 1987, Oroskar & Read, 1987, Oroskar & Read, 1989, Read *et al.*, 1993, Strom & Frenkel, 1987). The destabilization of viral mRNAs is thought to aid in sharpening the transition between sequential phases of viral gene expression by tightly coupling changes in transcription with mRNA levels (Kwong & Frenkel, 1987, Oroskar & Read, 1989, Read *et al.*, 1993, Strom & Frenkel, 1987).

The early shutoff of host protein synthesis or virion host shutoff is mediated by a structural component of the virion, and as such, does not require *de novo* synthesis of viral proteins to function. Four lines of evidence support this conclusion. First, viruses which were irradiated with UV light, still induce the shutoff of host protein synthesis and the disaggregation of polyribosomes (Fenwick & Clark, 1982, Fenwick & McMenamin, 1984, Fenwick & Walker, 1978, Nishioka & Silverstein, 1978). Second, cellular mRNA degradation still occurs upon HSV infection in the presence of drugs that inhibit either *de novo* transcription or translation (Fenwick & McMenamin, 1984, Fenwick & Walker, 1978, Schek & Bachenheimer, 1985, Strom & Frenkel, 1987). Third, the inhibition of host protein synthesis also occurs during HSV infection of cytoplasts which had their nucleus removed (Fenwick & Walker, 1978). Fourth, infection of cells by L-particles (contain tegument and envelope, but lack nucleocapsid) leads to host shutoff (McLauchlan *et al.*, 1992).

The viral gene responsible for the early shutoff of protein synthesis was found by mapping the *vhs*1 mutation to the UL41 open reading frame (Fenwick & Owen, 1988, Kwong *et al.*, 1988). The role of UL41 in early shutoff was confirmed by the construction viral mutants in which the UL41 gene was disrupted, and these mutants showed a deficiency in the ability to shutoff host protein synthesis and mediate mRNA degradation (Fenwick & Everett, 1990a, Read *et al.*, 1993, Smibert & Smiley, 1990). Additionally, HSV-2 displays a stronger shutoff phenotype than HSV-1 (Powell & Courtney, 1975) and when

the UL41 gene was taken from HSV-2 and inserted into HSV-1, HSV-1 with UL41(HSV-2) displayed a strong shutoff phenotype (Fenwick & Everett, 1990b). This further confirms that the UL41 gene product is responsible for early shutoff.

The UL41 gene is predicted to produce a primary translation product of 55 kDa (McGeoch *et al.*, 1988). This closely agrees with an observation that the *in vitro* translation product of the UL41 gene is a protein of 58 kDa (Anderson *et al.*, 1981) (Frink *et al.*, 1981). The protein encoded by UL41, vhs, was confirmed to be 58 kDa in infected cells, by the use of an antisera raised against a synthetic peptide which corresponded to a region of the predicted amino acid sequence of vhs (Smibert *et al.*, 1992), and an antisera raised against a UL41-*lac*Z fusion protein (Read *et al.*, 1993). The 58 kDa vhs protein is the major polypeptide, however a larger, 59.5 kDa protein was also detected in infected cells, although in lower abundance (Read *et al.*, 1993). Both proteins were phosphorylated, and the difference in size was due to the extent of the phosphorylation (Read *et al.*, 1993). Only the 58 kDa protein was detected in HSV-1 virions, confirming vhs as a virion component (Read *et al.*, 1993, Smibert *et al.*, 1992). vhs was also shown to be a part of the tegument, as it was present in deenveloped preparations of HSV L-particles (McLauchlan *et al.*, 1992).

The viral protein kinase UL13 has been implicated in virion-associated host shutoff. Viruses with a disrupted UL13 gene were unable to mediate shutoff of host protein synthesis (Overton *et al.*, 1994). In the original report by Overton *et al.* (Overton *et al.*, 1994), lower levels of vhs were detected in cells infected with UL13-negative mutants, however, there did not appear to be any difference in the amount of vhs incorporated into the virions (Overton *et al.*, 1994). A later report found that while UL13 or ICP22 deficient viruses were unable to shutoff host protein synthesis, there was much less vhs produced in infected cells and incorporated into virions, as compared to the wild-type virus (Ng *et al.*, 1997). Thus, the lack of host shutoff in UL13 or ICP22 deficient viruses is most likely due to the much lower amount of vhs initially delivered into infected cells, and reflects a requirement of ICP22 or the posttranslational modification of ICP22 for

efficient UL41 gene expression (Ng *et al.*, 1997). In addition, vhs was shown to be the only viral protein necessary and sufficient to mediate the shutoff of host protein synthesis. A *vhs* expression vector transfected into cells, was able to suppress the expression of cotransfected β-galactosidase (Jones *et al.*, 1995) and chloramphenicol acetyl transferase (Pak *et al.*, 1995) reporter genes.

The activity of the vhs delivered by the infecting virion is down regulated by one or more newly synthesized viral proteins, allowing viral mRNAs to accumulate after cellular mRNAs have been degraded (Fenwick & Everett, 1990a, Fenwick & Owen, 1988, Smibert & Smiley, 1990). The transactivator VP16 is the viral protein believed to be responsible for the down regulation of vhs activity. VP16 binds directly to vhs, both *in vivo* and *in vitro* (Smibert *et al.*, 1994). VP16 null mutants undergo accelerated host and viral mRNA turnover, and arrest of viral protein synthesis at intermediate times post-infection (Lam *et al.*, 1996), and an inactivating deletion in the UL41 gene eliminates this phenotype (Lam *et al.*, 1996). As well, a mutant VP16 protein which is unable to interact with vhs is able to complement the growth of a VP16/vhs null mutant but not a VP16 null virus, suggesting that direct interaction between VP16 and vhs is required for efficient virus growth (Knez *et al.*, 2003).

Although vhs is not essential for virus replication, vhs mutants do display a 5- to 10-fold reduction in virus yield in tissue culture infection (Read *et al.*, 1993, Read & N., 1983, Smibert & Smiley, 1990). In addition, vhs plays a critical role *in vivo* in HSV pathogenesis. Viral isolates bearing inactivating mutations in the UL41 gene display a greatly impaired ability to replicate in mouse corneas, trigeminal ganglia, and brains and a severely impaired ability to establish latency (Leib *et al.*, 1999, Strelow *et al.*, 1997, Strelow & Leib, 1995, Strelow & Leib, 1996). The basis for this profound attenuation remains unclear. However, accumulating evidence suggests that vhs plays a key role in disarming host innate and adaptive immunity and it is possible that these effects contribute to the attenuation of vhs mutants. Thus, vhs, along with ICP47, reduces the expression of MHC class I molecules thereby contributing to the resistance of

lysis of HSV infected cells by CTLs (Tigges *et al.*, 1996). vhs also aids in reducing the expression of MHC class II molecules (Trgovcich *et al.*, 2002) and prevents dendritic cell activation in response to viral infection (Samady *et al.*, 2003), thus reducing the amount of antigen presentation to the immune system. vhs has also been found to suppress production of the cytokines IL-1β, IL-8 and MIP-1α in macrophages and IL-8 in HEL cells (Suzutani *et al.*, 2000). Additionally, the vhs protein of HSV-2 but not HSV-1 has been found to interfere with the IFN α/β mediated innate antiviral response (Murphy *et al.*, 2003).

Strong evidence suggests that vhs is either a ribonuclease (RNase) or a subunit of a RNase which contains the active site. First, extracts of HSV-infected mammalian cells and extracts of partially purified virions display RNase activity, and this activity is eliminated by vhs mutations (Krikorian & Read, 1991, Sorenson *et al.*, 1991, Zelus *et al.*, 1996). Second, antibodies against vhs inhibit the RNase activity found in extracts of partially purified virions (Zelus *et al.*, 1996). Third, vhs induces endoribonucleolytic cleavage of a number of RNA substrates when it is expressed as the only HSV protein in the rabbit reticulocyte lysate (RRL) *in vitro* translation system (Elgadi *et al.*, 1999, Elgadi & Smiley, 1999, Zelus *et al.*, 1996). Fourth, vhs, along with homologues from other alphaherpesviruses shares amino acid sequence similarity in two regions with a family of mammalian, yeast, bacterial and phage nucleases involved in DNA replication and repair (Doherty *et al.*, 1996, Everly & Read, 1997, Everly *et al.*, 2002). These similarities appear to be functionally relevant, as mutations in vhs that alter conserved residues in the active site and essential for catalytic activity of cellular homologues, abrogated the ability of the vhs mutants to inhibit the expression of a reporter gene (Everly *et al.*, 2002). Lastly, Everly *et al.* partially purified a soluble complex of vhs and the eukaryotic translation initiation factor eIF4H when both were co-expressed in *E coli.* and showed that it displays RNase activity (Everly *et al.*, 2002). The RNase activity of the complex was eliminated by mutations that alter residues located in the nuclease motif (Everly *et al.*, 2002), suggesting that vhs contains at least in part, the active site. Taken

together, these data indicate that vhs is an integral and required part of the vhs-dependent RNase. However, vhs has not yet been purified to homogeneity in a soluble and biologically active form. Thus, the possibility remains that the vhs-dependent RNase requires one or more cellular factors such as eIF4H for full activity.

Most if not all viral and cellular mRNAs are targeted for degradation by vhs, while rRNAs and tRNAs are spared both *in vitro* and *in vivo* systems (Krikorian & Read, 1991, Kwong & Frenkel, 1987, Oroskar & Read, 1989, Zelus *et al.*, 1996). Thus, mRNAs may be selected for degradation via a common feature not present in other RNAs. The 3' poly(A) tail was originally proposed as a recognition site for the vhs-dependent RNase (Zelus *et al.*, 1996), however in both the RRL system and in extracts of HSV-infected cells it has been observed that the poly(A) tail is not required for vhs-dependent degradation of an RNA substrate (Elgadi *et al.*, 1999, Karr & Read, 1999). The presence of a 5' cap was also found to be dispensable for vhs-dependent degradation of mRNAs in both RRL and extracts of partially purified virions (Elgadi *et al.*, 1999, Zelus *et al.*, 1996). Although the foregoing might be taken to exclude roles for both the 5'-cap and 3' poly(A) tail, it is important to note that neither modification is required for efficient translation in RRL. Thus, either/or both features may be important for substrate recognition *in vivo*. Indeed, several lines of evidence suggest that vhs targets mRNAs via interactions with the translation apparatus. In HSV infected cells, sequences at the 5' end of HSV thymidine kinase mRNA are degraded before the sequences at the 3' end of the transcript (Karr & Read, 1999). Consistent with this observation, the initial cleavage sites of signal recognition particle α-subunit (SRPα) mRNA are clustered around the 5' end in RRL containing pretranslated vhs (Elgadi *et al.*, 1999). As well, the internal ribosome entry site (IRES) of the encephalomyocarditis virus (EMCV) and poliovirus serve as movable elements which direct vhs-dependent cleavage events to be clustered immediately 3' downstream of the IRES (Elgadi & Smiley, 1999). However, the degradation of mRNA in cells may be complicated, as a

recent report found that in HSV infected cells the partially degraded IEX-1 RNA lacked the 3' sequences rather than the 5' sequences (Taddeo *et al.*, 2003).

In most cases, the initial cleavage sites appear to be near areas of translation initiation, raising the possibility that vhs may interact with a component(s) of the translation initiation machinery. Consistent with this hypothesis, vhs has been found to interact with the eukaryotic translation initiation factor eIF4H in mammalian cells (Feng *et al.*, 2001). Some vhs mutants which were unable to degrade RNA *in vivo* were also unable to interact with eIF4H (Feng *et al.*, 2001), suggesting the interaction is important for vhs activity. eIF4H is a 25 kDa protein which stimulates the translational activity of the eIF4B dimer (160 kDa) and eIF4F (composed of eIF4E, eIF4A and eIF4G) and stimulates the helicase activity of eIF4A(46 kDa) by increasing the processivity of eIF4A (Richter *et al.*, 1999, Richter-Cook *et al.*, 1998, Rogers *et al.*, 1999, Rogers *et al.*, 2001). eIF4H is thought to work together with eIF4A and eIF4B to unwind secondary structure in the 5' untranslated regions of mRNAs. eIF4B, the sequence paralogue of eIF4H functions to stimulate the RNA binding, ATPase, (reviewed in(Rogers *et al.*, 2001)), and together with eIF4H modulates the helicase activity of eIF4A and eIF4F (Rogers *et al.*, 2001). For more detail of translation initiation see the section below.

Everly *et al.* (Everly *et al.*, 2002) have shown that the vhs/eIF4H complex displays detectable RNase activity in the absence of other viral and host proteins. However, it is not yet clear whether vhs itself has RNase activity. In addition, the role of eIF4H and other cellular factors in controlling vhs activity and targeting to mRNAs has not yet been assessed. Possibly relevant to these questions, a previous report from this laboratory showed that extracts of yeast engineered to express vhs displayed little if any RNase activity (Lu *et al.*, 2001a). However, vhs-dependent RNase activity could be restored with the addition of unprogrammed RRL (Lu *et al.*, 2001a), suggesting that the vhs-dependent RNase requires one or more cellular factors for efficient activity.

1.7 Translation Initiation

Two modes of translation initiation on mRNAs have been defined. Cap-dependent initiation is used by the majority of cellular mRNAs (reviewed in (Hellen & Sarnow, 2001, Pestova *et al.*, 2001), Figure 1.1). The first stage of translation initiation involves the binding of the eukaryotic initiation factor 2 (eIF2), initiation tRNAs, eIF1A, and eIF3, to the 40S ribosomal subunit to form a 43S complex. The second stage is the binding of the 43S ribosomal complex to the 5' end of the mRNA and involves the translation initiation factors eIF3, eIF4A, eIF4B, eIF4F and the poly (A)-binding protein (PABP). eIF4F is composed of eIF4E, the cap binding protein, eIF4G, the scaffolding/adapter protein, and eIF4A, the ATPase/RNA helicase. eIF4F binds the 5' end of the mRNA through the recognition of the 5'-terminal m^7G cap by eIF4E. eIF4A in conjunction with eIF4B and eIF4H, unwinds secondary structure in the 5' untranslated region of the mRNA. The N-terminal of eIF4G binds eIF4E and PABP (Imataka *et al.*, 1998, Mader *et al.*, 1995), the middle portion interacts with eIF3, eIF4A and RNA (Imataka & Sonenberg, 1997, Lamphear *et al.*, 1995) (Morino *et al.*, 2000, Pestova *et al.*, 1996b), and the C-terminal of eIF4G contains another binding site for eIF4A (Imataka & Sonenberg, 1997, Lamphear *et al.*, 1995, Morino *et al.*, 2000). The binding of 43S complexes to mRNA is thought to be mediated through the interactions of eIF4G and eIF4B (which also interacts with eIF3) with ribosome bound eIF3 (Lamphear *et al.*, 1995, Methot *et al.*, 1996a). The ribosomal complex then scans to the AUG codon and the 60S subunit joins to form an active 80S ribosome and polypeptide elongation begins.

A second mode of translation initiation used by some cellular and viral mRNAs is cap-independent internal initiation, mediated by internal ribosome entry sites (IRES) (reviewed in (Hellen & Sarnow, 2001, Pestova *et al.*, 2001)). Picornaviruses are a family of viruses that are non-enveloped and have a positive-strand RNA genome. Picornavirus 5'-untranslated regions are very long, have many AUG triplets and are predicted to contain complex secondary structure. Picornavirus IRES structures are divided into three groups: cardio-

and aphthoviruses, hepatitis A virus and entero- and rhinoviruses. IRES elements were originally discovered in poliovirus (Pelletier *et al.*, 1988) and EMCV (Jang *et al.*, 1988) and were subsequently found in many other viral and cellular mRNAs (reviewed in (Martinez-Salas *et al.*, 2001)). Initiation by direct internal ribosome entry was shown by inserting the viral 5' non-translated region between the two cistrons of a bicistronic mRNA, and finding that this lead to an enhancement of expression of the downstream cistron (Jang *et al.*, 1988, Pelletier *et al.*, 1988). Infection of cells by picornaviruses leads to a rapid inhibition of cap-dependent translation. Enteroviruses such as poliovirus, and rhinoviruses produce proteases which cause the cleavage of eIF4G into an N-terminal one third fragment, which contains the eIF4E and PABP binding sites, and a C-terminal two-thirds fragment which contains the eIF3 and both eIF4A interaction sites (Lamphear *et al.*, 1995). On the other hand, infection by cardioviruses, such as EMCV, does not cause the shutoff of host protein synthesis by cleavage of eIF4G. Rather, they cause the dephosphorylation of 4E-BPs, which then bind eIF4E and prevent it from entering the eIF4F complex, thus resulting in an inhibition of cap-dependent translation (Gingras *et al.*, 1996).

Viral IRESs are the best characterized and show substantial diversity in their mechanism of action. They are defined solely by functional criteria and there are no significant similarities between individual IRESs unless they are from related viruses. The EMCV IRES is among the best understood. It is ~450 nucleotides long and contains highly structured domains which lie upstream of the initiation codon. Initiation of translation on the EMCV IRES does not involve scanning, and ribosomal complexes are targeted directly to the initiation codon which is located at the 3' border of the IRES (Kaminski *et al.*, 1990). EMCV IRES-mediated initiation is ATP-dependent and the assembly of 48S ribosomal complexes on the IRES requires eIF2, eIF3, eIF4A and the C-terminal two thirds of eIF4G (which eIF4A binds), but does not require any IRES *trans*-acting factors (Pestova *et al.*, 1996a, Pestova *et al.*, 1996b). The translation factor

eIF4B and the pyrimidine tract binding-protein (PTB) enhance the assembly of 48S ribosomal complexes on the IRES, but are not an absolute requirement (Pestova *et al.*, 1996a). eIF4G binds to the J-K domain (EMCV bases 682-795) of the IRES, and recruits eIF4A and eIF4B to the IRES (Kolupaeva *et al.*, 1998, Pestova *et al.*, 1996b). All three factors can bind the IRES directly, but the presence of all factors enhances the binding of all three to the IRES (Kolupaeva *et al.*, 1998, Pestova *et al.*, 1996b). The interaction of eIF4A with eIF4G enhances the affinity of the eIF4A/eIF4G complex for the IRES (Lomakin *et al.*, 2000). This complex is essential to recruit the 43S ribosomal complex to the IRES. The ribosome binds directly at or very close to the initiation codon, without scanning from the 5' end of the mRNA and translation begins from AUG-11 (Kaminski *et al.*, 1994, Kaminski *et al.*, 1990).

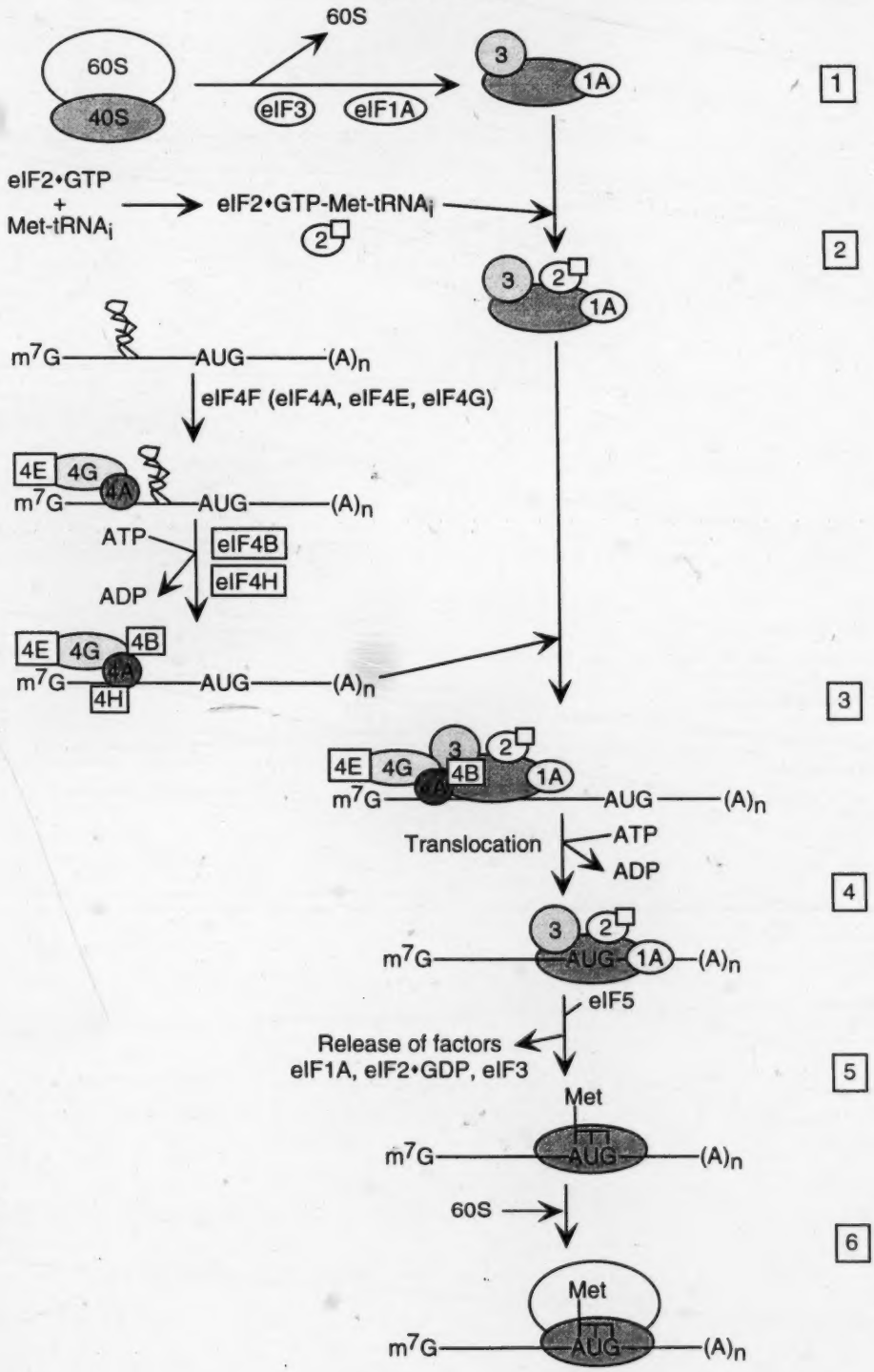
In contrast, the hepatitis C virus (HCV) IRES binds the 40S ribosomal subunit in the absence of initiation factors, in such a way that the ribosomal peptidal site is in the immediate proximity of the initiation codon (Pestova *et al.*, 1998). The addition of ternary eIF2/GTP/initiator tRNA complex is sufficient for the bound 40S subunit to lock onto the initiation codon (Pestova *et al.*, 1998). The cricket paralysis virus (CrPV) IRES is another type of IRES, in that 80S/CrPV IRES complexes can be formed without Met-tRNA, eIF2 or GTP hydrolysis (Wilson *et al.*, 2000). The IRES itself subsitutes functionally for the role of initiator tRNA (Wilson *et al.*, 2000). IRES elements have also been found in an number of cellular mRNAs which encode proteins that are involved in growth control and cellular stress responses, such as hypoxia and apoptosis (reviewed in (Hellen & Sarnow, 2001, Martinez-Salas *et al.*, 2001)).

1.8 Thesis Rational

When I started this project it was known that eIF4H could interact with vhs (Feng *et al.*, 2001), however the effect of eIF4H on the activity and targeting of vhs was unknown. From work by Lu *et al.*, it was clear that the vhs produced in yeast required one or more mammalian factors for efficient activity (Lu *et al.*,

2001a). Therefore, I tested whether eIF4H could be the mammalian factor that stimulated the activity of vhs expressed in yeast. Specifically I sought to determine whether eIF4H contributes to the RNase activity of the vhs-dependent RNase or the targeting of vhs to specific sites on RNA substrates. In order to address this question, I partially purified the translation initiation factors eIF4A, eIF4B and eIF4H, and assayed them for their ability to enhance the RNase activity of extracts of yeast that expressed vhs. During the course of my investigation, a new report was published that showed that a complex of vhs and eIF4H displayed RNase activity (Everly *et al.*, 2002). However, since they were unable to purify vhs to homogeneity in a soluble form, the role of eIF4H in this complex, whether it was to enhance the RNase activity or target vhs to mRNAs, was still unclear. The results from my work show that both eIF4H and its sequence paralogue eIF4B interact with vhs and can stimulate the RNase activity of vhs produced in yeast. However, none of the translation initiation factors tested could restore IRES-mediated targeting to vhs, indicating that one or more other cellular factors is required to restore targeting to vhs.

Figure 1.1: Mechanism of translation initiation. This figure represents the current working model for translation initiation. Step 1: initiation begins with the dissociation of a preexisting 80S ribosome to the individual subunits, followed by the binding of eIF3 and eIF1A to the 40 S subunit. Step 2: Met-tRNA$_i$ binds to eIF2•GTP to form a ternary complex which binds to the 40S subunit to form the 43S preinitiation complex. Step 3: eIF4F (composed of eIF4A, the RNA helicase, eIF4E, the cap-binding protein, and eIF4G, the scaffolding protein) binds to the m^{7}G-cap. Secondary structure near the 5' end is removed by eIF4F (or eIF4A), in combination with eIF4B and eIF4H, and requires the hydrolysis of ATP. The 43S complex is then binds to the mRNA through an interaction between eIF4G and eIF3 (bound to the 40S subunit). Step 4: The 40S complex scans along the 5' UTR, in an ATP-driven process, until it encounters an initiation codon (AUG) in a favorable sequence context. Step 5: When the start codon is reached by the 40S initiation complex, the associated initiation factors are released. This process is mediated by eIF5, which is a GTPase-activating protein, and promotes GTP hydrolysis by eIF2. Step 6: Once the initiation factors have disassociated, the 60S subunit binds to the 40S subunit and polypeptide elongation begins.


60S
60S
40S
eIF3
eIF1A
3
1A
1
eIF2•GTP
+
Met-tRNA$_i$
eIF2•GTP-Met-tRNA$_i$
2
2
3
2
1A
m^{7}G
AUG
(A)$_n$
eIF4F (eIF4A, eIF4E, eIF4G)
4E
4G
4A
ATP
ADP
eIF4B
eIF4H
4B
4H
3
4E
4G
4B
4A
3
2
1A
Translocation
ATP
ADP
4
eIF5
Release of factors
eIF1A, eIF2•GDP, eIF3
5
Met
60S
6
Met

CHAPTER 2 : MATERIALS AND METHODS

2.1 PLASMIDS

The eIF4H open reading frame (ORF) was obtained by RT-PCR from HeLa cell RNA using the following PCR primers: OSW1 (5'-GACGGCATATGGCGGACTTCGACC-3'), used to engineer a *Nde*I site at the AUG start codon to clone eIF4H in frame into pET15b (Novagen) and OSW2 (5'-CTCGACTCCCTCCCAACCGCAGGCTCATTC-3'), used to engineer a *Xho*I site after the stop codon (Figure 2.1). The PCR products were ligated into pGEM-T Easy vector (Promega). Both eIF4H and eIF4H$_i$ (contains a 20 amino acid insertion after residue 137) were isolated creating plasmids pSW1 and pSW2 respectively (performed by Shawn Wasilenko, as a rotation student in our lab). The sequence of eIF4H$_i$ was pristine, however the sequence coding for eIF4H contained a deletion of one cytosine in a run of seven cytosines in positions 531-537. To correct the deletion, oligonucleotide directed mutagenesis was used. eIF4H was cut out of pSW1 using *Sph*I and *Sac*I and ligated into pSELECT-1 (Altered Sites Mutagenesis Kit – Promega). The mutagenic oligo (5'-GGCTGCCCATGGGGGGGCCTGTTCGCC-3') was annealed to the recombinant ssDNA template. T4 DNA Polymerase was used to synthesize the mutant strand and the ligated plasmid was transformed into BMH71-18 *mut*S. DNA was isolated and retransformed into HB101. Mutants were sequenced and one with the mutation corrected was designated pGC2 (performed by Gary Chan, as a rotation student in our lab).

pET15b (Novagen) is a bacterial expression vector that encodes a His_6-tag at the N-terminus and a *Nde*I site after the tag to clone the insert in frame. The *Nde*I fragment of pGC2 bearing the eIF4H ORF was cloned into the *Nde*I site of pET15b creating pRD1 (Figure 2.1). To clone eIF4H$_i$ into pET15b, pSW2 was digested with *Sph*I, the ends were made flush with T4 DNA polymerase

and then the plasmid was digested with *Nde*I. The *Sph*I- *Nde*I fragment bearing the eIF4H$_i$ ORF was cloned into the *Nde*I-*Bam*HI site of pET15b (after repairing the *Bam*HI site with the Klenow fragment of DNA polymerase I) to generate pRD2.

Gene Storm Expression Ready clones that contained the ORF of eIF4A (accession no. D13748) or eIF4B (accession no. X55733) were purchased from Invitrogen. To clone eIF4A into pET15b (Figure 2.2), the eIF4A ORF was PCR amplified (PlatinumTaq High Fidelity; Invitrogen) from pCDNA3.1/GS/eIF4A (Invitrogen) using PCR primers: ORD4 (5'-CATATGTCTGCGAGCCAGCAT-3'), used to engineer a *Nde*I site at the start codon, and ORD5 (5'-TCAGATGAGGTCAGCAACATTGAGG-3'), used to create a stop codon at the C-terminus of the protein. The eIF4A PCR product was ligated into pCR2.1-TOPO (Invitrogen) to generate pRD6. The eIF4B ORF was PCR amplified as described for eIF4A using PCR primers: ORD1 (5'-CATATGGCGGCCTCAGCAAAAAAG-3'), and ORD14 (5'-TTACTATCATTCGGCATAATCTTCTCCCTC-3'), and cloned into pCR2.1-TOPO (Invitrogen) creating pRD9 (Figure 2.2). The *Nde*I-*Bam*HI fragments of pRD6 and pRD9 containing the ORFs of eIF4A and eIF4B, respectively, were cloned between the *Nde*I-*Bam*HI sites of pET15b to generate pRD12 and pRD10, respectively.

DNA primers were synthesized using the Applied Biosystems 394/8 synthesizer and plasmids were sequenced usingf a Beckman Coulter CEQ2000XL DNA sequencing system at the Biochemistry DNA Services Laboratory, Faculty of Medicine, University of Alberta.

pcdc34Δ_{209}His is a bacterial expression plasmid that expresses the yeast ubiquitin-conjugating enzyme cdc34Δ_{209} (Ptak *et al.*, 1994), generously supplied by Michael Ellison(University of Alberta).

The *in vitro* transcription vectors pCITE-1 and pSPSR19N which are used to generate the reporter RNA substrates, pCITE-1 and SRPα, respectively and

the vhs *in vitro* translation vector pSP6vhs have been previously descibed (Elgadi *et al.*, 1999, Elgadi & Smiley, 1999).

The yeast expression vectors pYEX-BX (Clontech) and pYEX-BX2.1vhs which encodes the 2.1vhs ORF have been previously described (Lu *et al.*, 2001a). The 2.1vhs ORF contains a 8xHis tag after codon 138 and a HA-tag after condon 344 (Elgadi *et al.*, 1999).

2.2 Bacterial Strains and Growth Conditions

All plasmids, unless otherwise stated, were maintained and amplified in *Escherichia coli* strain HB101 (F⁻Δ[*gpt-proA*]62 *leuB*6 *supE*44 *ara*-14 *galK*2 *lacY*1 Δ[*mcrC-mrr*] *rpsL*20 [Str^r] *xyl*-5 *mtl*-1 *recA*13) (Maniatis *et al.*, 1989). Plasmids derived from pCR2.1-TOPO were maintained and amplified in strain TOPO10 Electrocomp (Invitrogen) (F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lacX*74 *rec*A1 *deo*R *ara*D139 Δ(*ara.leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G). Plasmids derived from pCDNA3.1/GS were maintained and amplified in strain GeneHogs (Invitrogen) (F⁻ mcrA Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lacX*74 endA1 *rec*A1 *deo*R *ara*D139 Δ(*ara.leu*)7697 *gal*U *gal*K *rps*L *nup*G). *E.coli* strain BMH17-18*mut*S (Promega) (*thi sup*E Δ(*lac-pro*AB) [*mut*S::Tn10] [F' *pro*A$^+$B$^+$ *laq*I^{q}ZΔM15]) was used in oligonucleotide mutagenesis. All protein expression was preformed in strain BL21(DE3)pLysS (Novagen) (F⁻ *dcm ompT hsd*S(r_B^- m_B^-) *gal* λ(DE3) [pLysS Cam^r]).

All strains transformed with recombinant plasmids were cultured at 37°C in Luria-Bertani medium (LB; 1.0 % Bacto Tryptone, 0.5 % yeast extract, 1.0 % NaCl, pH 7.0) containing 100 μg ampicillin per ml in a shaker incubator set at 225rpm. Chloramphenicol (34 μg/ml) was also added when culturing strain BL21(DE3)pLysS. For the GeneHogs strain, the salt concentration of the LB was reduced to 0.5 %, the pH increased to pH 7.5 and the antibiotic zeocin (25 μg/ml; Invitrogen) was used.

2.3 Yeast Strain, Growth Conditions, and Yeast Transformation

The *Saccharomyces cerevisiae* strain W303-1A(MAT**a** SUC3 ade2-1 can1-100 his3-11,*15* leu2-3,112 trp1-1 ura3-1), (Livingstone-Zatchej *et al.*, 1997) was used as the host for plasmids derived from pYEX-BX. Growth conditions and transformation of W303-1A have been described previously (Lu *et al.*, 2001a).

2.4 Preparation of Yeast Extracts for the Vhs Activity Assay

The method of Schultz *et al.* (Schultz *et al.*, 1991, Schultz *et al.*, 1997) was used for the preparation of frozen cells and yeast extracts. Briefly, one litre of YNBG (0.67 % yeast nitrogen base without amino acids, 2 % galactose; supplemented with adenine, histidine and tryptophan) was inoculated with an overnight culture of *S. cerevisiae* strain W303-1A/pYEX-BX or W303-1A/pYEX-BX2.1vhs and grown to an OD_{600} of ~ 3-4. The culture was induced with 0.15 mM $CuSO_4$ for 5 hr. The cells were then harvested, washed, frozen in liquid nitrogen and stored at -80°C. About 3 g of frozen cells were used to prepare the extracts. Frozen cells were ground in a coffee mill, thawed and resuspended in 1.3 volumes of extraction buffer (100 mM HEPES-KOH, pH 7.9, 245 mM KCl, 5 mM EGTA, 2.5 mM DTT) with protease inhibitors. The extract was centrifuged at 100,000 Xg for 2hrs. The supernatant was then collected and dialyzed into vhs assay buffer (1.6 mM Tris-acetate, 80 mM potassium acetate, 2.0 mM magnesium acetate, 0.1 mM DTT). Aliquots were frozen and stored at -80°C. ATP (0.25 mM) and 1µl of RNaseOut (Invitrogen) per 50µl sample were added upon thawing of aliquots.

2.5 Protein Expression and Purification

Protocols were adapted from "The QIAexpressionist" (Qiagen). For the expression of eIF4H, $eIF4H_i$ and $cdc34\Delta_{209}$, one liter of culture was grown to an OD_{600} of ~ 0.3-0.4 and then induced with 1 mM IPTG for 3 hrs. The cells were

then harvested, resuspended in 2ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, 25 % glycerol, pH adjusted to 8.0 with NaOH) per gram of wet weight, and frozen at -80°C. All subsequent purification steps were performed at 4°C or on ice. Cells were thawed for 30 min and lysed by three sonication cycles of 30 sec each at setting 7 (550 Sonic Dismembrator with cup; Fisher Scientifc). The lysate was incubated with DNaseI (5 µg/ml) and 8 mM $MgCl_2$ for 20 min and the insoluble material was removed by centrifugation for 25 min at 10,000 Xg. The supernatant was incubated with 1ml of Ni-NTA agarose (Qiagen) per 4ml lysate for 1hr on a Nutator (Becton Dickinson). The resin was pelleted and washed sequentially with 10 ml lysis buffer, wash buffer (lysis buffer + 20mM imidazole), and lysis buffer. The protein was batch-eluted twice with 1X the vol of resin of elution buffer (lysis buffer + 400mM imidazole). Elution fractions were combined and dialyzed overnight into DEPC-treated vhs assay buffer with 25 % glycerol or Buffer B (20 mM Tris, 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, 2.0 mM $MgCl_2$) with 25 % glycerol. Aliquots were frozen at -80°C. ATP (0.25mM) and RNaseOut (Invitrogen) were added after thawing of aliquots. We obtained 3-4 mg of eIF4H and 1-1.5 mg of cdc34Δ_{209} per litre of culture. eIF4H was as active in Buffer B as in vhs assay buffer.

eIF4A and eIF4B were expressed and purified in the same manner as eIF4H, with a few modifications. Two liters of culture per protein purified were grown to an OD_{600} of 1.0 and induced with 1 mM IPTG for 2 hrs. Cells expressing eIF4B were grown at 30°C instead of 37°C. Cells were harvested, resuspended in lysis buffer (10 % glycerol) and lysed as described above. One ml of Ni-NTA agarose was added per 6 ml lysates expressing eIF4B. Bound protein was batch-eluted with 1/2 X the vol of resin of elution buffer (10 % glycerol). Elution fractions were dialyzed overnight into DEPC-treated Buffer B with 10 % glycerol. Aliquots were frozen and stored at -80°C. ATP (0.25 mM) and 1 µl RNaseOut per 50 µl protein were added upon thawing of aliquots. Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad) using BSA as the standard.

2.6 Western Blot

Samples indicated were run on a 12 % SDS-PAGE gel and transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia), at 400 milliAmps for 45 min. After the transfer was complete the blot was blocked in 10 % skim milk TBS-Tween (25 mM Tris, pH 8, 150 mM NaCl, 0.1 % Tween-20) overnight at 4 °C. The blot was washed 3X 5 min with TBS-Tween and incubated with the primary antibody, anti-tetra-His antibody (Quiagen) at a dilution 1/2000 or anti-HA High Affinity (Roche) at 1/1000, for 1 hr at room temperature. The blot was washed 3X 10 min with TBS-Tween and incubated with the secondary antibody, anti-mouse horseradish peroxidase at a dilution of 1/3000 or anti-rat Ig-POD, Fab fragments (Roche) at 1/1000, for 1/2 hr at room temperature. The blot was washed 3X 10 min with TBS-Tween and developed with ECL+plus system (Amersham Biosiences) according to the manufacture's protocol. The signal was visualized using Fuji Super RX X-Ray film.

2.7 *In Vitro* Transcription and RNA Labeling

vhs mRNA to be used for *in vitro* translation was produced using the plasmid pSP6vhs (Elgadi *et al.*, 1999). Ten micrograms of *Eco*RI-linearized pSP6vhs template DNA was purified using a QIAquick column (Qiagen) according to the manufacturer's protocol (Elgadi *et al.*, 1999, Smiley *et al.*, 2001). The 20 µl *in vitro* transcription reaction contained 1 µg of linear template, 0.5 mM cap primer $m^7G(5')ppp(5')G$ (Pharmacia), 40 U of RNaseOut (Invitrogen), 0.25 mM of ATP, CTP, GTP and UTP, and 40 U of SP6 RNA polymerase (Invitrogen) in the buffer provided by the manufacturer of the polymerase. The reaction proceeded for 1 hr at 37°C, after which the template was degraded by 10 U of RNase-free DNase I (Ambion) for 20 min at 37°C. The RNA was cleaned by phenol-chloroform extraction, and precipitated with 95%

ethanol. The RNA pellet was washed sequentially with 70% ethanol, 95% ethanol, and then resuspended in 12 µl of RNase-free water.

Uncapped, internally labeled reporter RNAs were generated by the method of Elagdi and Smiley (Elgadi & Smiley, 1999, Smiley *et al.*, 2001). To generate pCITE reporter RNA, an *Eco*NI- linearized template DNA (pCITE-1) was transcribed with T7 RNA Polymerase to yield a runoff transcript of 2.3 kb (Elgadi & Smiley, 1999). SRPα reporter RNA was transcribed using an *Eco*RV-linearized template DNA (pSPSR19N) and SP6 RNA Polymerase to yield a runoff transcript of 2.4 kb (Elgadi *et al.*, 1999). The in vitro transcription reaction was preformed as described above except that the GTP concentration was reduced to 0.125 mM, the cap primer was omitted, 1 µCi of [α-^{32}P] GTP (3000Ci/mmol, New England Nuclear) was added and the reaction was shortened to 40 min. The transcription reaction was stopped by the addition of 2 µl of RNA loading buffer (50% glycerol, 1 mM EDTA, 10 mg/ml xylene cyanol, and 10 mg/ml bromophenol blue). The RNA was separated on a 1XTBE (90 mM Tris-borate, 2 mM EDTA) agarose gel containing ethidium bromide (1 µg/ml) at 100 V for 30 min. A gel slice containing the full-length transcript was excised from the gel using an RNase-free scalpel. The reporter RNAs were electroeluted from the gel slice into a 100 µl 7.5 M ammonium acetate trap in a six-well V-channel electroelutor (International Biotechnologies Inc.) at 100 V for 1 hr in 0.5XTBE. The RNA was recovered by ethanol precipitation and sequentially washed with 70% and 95% ethanol. The substrate RNAs were counted without scintillant in a Beckman LS6500 scintillation counter and resuspended to 4000 Cerenkov cpm/µl.

Unlabeled pCITE reporter RNA was generated using the MEGAscript transcription kit (Ambion) according to the manufacturer's protocol.

2.8 *In Vitro* Translation

In vitro translation of vhs RNA was preformed using the RRL system (Promega), according to the manufacturer's protocol as previously described

(Elgadi *et al.*, 1999, Smiley *et al.*, 2001). Briefly, 2 µl of the vhs RNA template (~ 2 µg) is incubated in 50 µl of RRL lysate supplemented with amino acids (minus methionine), 40 U of RNaseOut (Invitrogen), and 2 µl of [^{35}S] methionine (1175 Ci/mmol; New England Nuclear). Control reactions were preformed as described above except that the mRNA was omitted. The reactions were carried out for 90 min at 30°C and are stored at -80°C.

2.9 VHS ACTIVITY ASSAY

The vhs activity assay has been described elsewhere (Lu *et al.*, 2001a). Briefly, yeast extracts were mixed with various test proteins, blank RRL or buffer. The amount of total protein of yeast extract used per timepoint was 20 µg before the addition of test proteins. Reporter RNAs (4000 Cerenkov cpm per timepoint) were added to samples indicated and all reactions were adjusted to the same final volume with vhs assay buffer or Buffer B. The reactions were incubated at 30°C and aliquots were removed at indicated timepoints. RNA was recovered with the RNeasy Mini Kit (Qiagen) using the "clean up" protocol following the manufacturer's instructions. RNA samples were added to10 mM EDTA made with RNase-free water to a final volume of 100 µl. Buffer RLT (Qiagen) and 95 % ethanol were added and the mixture was added to an RNeasy Mini Spin column. The column was washed twice with wash buffer RPE (Qiagen) and eluted with 50 µl RNase-free water. The RNA was precipitated with 1 vol of isopropanol and 1/10 vol of 3 M sodium acetate. The RNA pellet was washed sequentially with 70 % and 95 % ethanol, and then resuspended in 25 µl RNA sample buffer (1 X MOPS buffer [200 mM MOPS, 50 mM sodium acetate, 5 mM EDTA, adjust pH to 7.0 with NaOH], 50 % deionized formamide, 16.7 % formaldehyde).

2.10 AGAROSE GEL ELECTROPHORESIS AND NORTHERN BLOT ANALYSIS

RNA samples were incubated at 55°C for 10 - 15 min and then transferred to ice for 2 min. RNA samples were combined with 2 µl RNA loading buffer (50 % glycerol, 1mM EDTA, 10 mg of xylene cyanol / ml, and 10 mg of bromophenol blue / ml) and loaded on to a 1.2 –1.4 % agarose gel containing 2 % formaldehyde. Electrophoresis was carried out in 1 X MOPS buffer at 130 volts for 2 hr until the gel had run ~ 7 – 8 cm. RNA was transferred to a GeneScreen Plus (NEN Life Sciences Products) nylon membrane in 10 X SSC (1.5 M sodium chloride, 150 mM sodium citrate) . Following UV-cross linking (Stratalinker 2400; Stratagene) ^{32}P-labeled RNA fragments were detected by exposure to Kodak BioMax MS film at - 80°C. The data was quantified by phosphorimager analysis on a Storm 860 (Molecular Dynamics) using the software program Image Quant for Macintosh version 1.2.

Unlabeled RNA fragments were detected by hybridization. After UV-cross linking, the membranes were rinsed in 2 X SSC and prehybridized in 15 ml of modified westneat solution (6.6 % SDS, 250 mM MOPS, pH 7.0; 5 X Denhardts solution , 1 mM EDTA) at 48°C for 1 hr. Membranes were hybridized to either ORD17 (5'-GCCTTATTCCAAGCGGCTTCGGCCAGTC-3'), complementary to residues 45 – 72 of the pCITE transcript, or JPP-E (5'-GCCGGAGTTGGATGATGACCCGACG-3'), complementary to the extreme 3' end of the pCITE RNA. Oligonucleotides were 5'-^{32}P labeled using T4 polynucleotide kinase (Invitrogen). The entire kinase reaction, after a 10 min incubation at 95°C, was added to the prehybridization westneat solution. Hybridization was carried out overnight at 48°C. The membrane was then washed twice in 2 X SSC – 0.2 % SDS at room temperature for 5 min and twice in 0.2 X SSC – 0.2 % SDS at 48°C for 20 min before being subjected to autoradiography.

2.11 Far Western Analysis

The far western protocol was preformed following the method of Guitchet *etal.* (Guichet *et al.*, 1997). Briefly, 8 µg of each test protein was run on a 12%

SDS-PAGE gel and transferred to a nitocellulose membrane (Hybond ECL membrane, Amersham Biosciences). Human Protein Phosphatase 1(PP1) was kindly provided by Kathleen Perrault (University of Alberta). After the transfer was complete, the blot was blocked with 2% milk powder in AC Buffer (10 % glycerol, 100mM NaCl, 20mM Tris, 0.5mM EDTA, 0.1 % Tween-20) for 1hr at 4°C. To prepare the probe, RRL containing ^{35}S-labeled vhs generated by *in vitro* translation was spun through a 1cc syringe packed with G-25 resin (Amersham Pharmacia) to remove unincorporated amino acids. The probe was mixed with 10 ml 2% milk powder in AC buffer with 1mM DTT. After the blocking step, the probe mix was added to the blot and incubated for 2 hr at 4°C. The blot was washed with 2 % skim milk in AC buffer for 15 min and 4X 20min in AC buffer. The blot was dried and visualized by autoradiography.

2.12 Helicase Unwinding Assay

The assay has been described elsewhere (Du *et al.*, 2002, Rogers *et al.*, 1999). Briefly, complementary RNA oligonucleotides: RNA_{25}-2 (5'-CACCGUAAAGCACGCAAAAACAAAA-3') and RNA_{11}-1 (5'-GCUUUACGGUG-3') were purchased from Dharmacon Research. RNA_{11}-1(2 µM) was ^{32}P-labelled using T4-kinase (Invitrogen). Duplexes were prepared by annealing unlabelled RNA_{25}-2 (15 pmole) with complementary labeled RNA_{11}-1 (7.5 pmole) in a 2:1 ratio in buffer containing 10 mM Tris, pH 7.5, 1mM EDTA, and 100 mM KCl. The mixture was heated to 95°C for 5 min then slowly cooled to 4°C over 90 min (0.1°C/5s). The duplex was diluted to 0.02 pmol/µl. Each unwinding reaction contained 25 mM HEPES, pH 7.5, 70 mM KCl, 2 mM DTT, 1mM ATP, 1mM $MgCl_2$, 2 nM labelled duplex, 1mg/ml BSA and 0.8 µM eIF4A in 20µl total. Duplex was added last and the reactions were incubated at 35°C for times indicated. The reactions were stopped by the addition of 5 µl stop buffer (50 % glycerol, 2 % SDS, 20 mM EDTA, 0.01 % bromphenol blue, 0.01 % xylene cyanol). The reaction products were resolved on a 18 % Tris borate–EDTA mini-

gel at 200 V for 1.5 hr at 4 °C. The gel was dried for 1 hr and visualized by autoradiography.

Figure 2.1: Diagram of the cloning strategy to create the eIF4H and eIF4Hi expression vectors. The eIF4H ORF was cloned into pGEM-T easy, to create pSW1, subcloned into pSelect-1 to generate pGC2 and finally into the expression vector pET15b to produce pRD1. The eIF4Hi ORF was cloned into pGEM-T easy to generate pSW2 and then into the expression vector pET15b to produce pRD2. See Materials and Methods for more details. Restriction sites are as follows: N - *Nde*I, S - *Sph*I, X -*Xho*I, Sa - *Sac*I, and B - *Bam*HI.

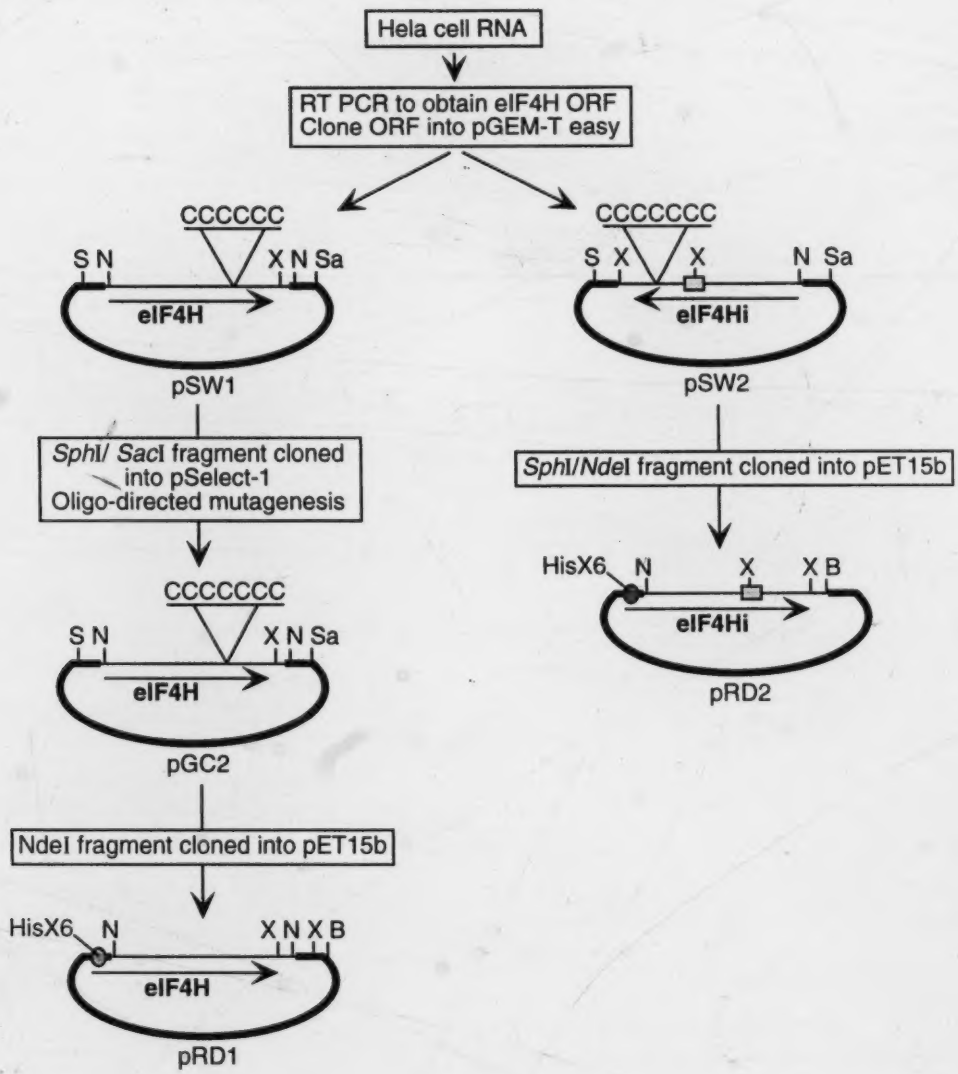
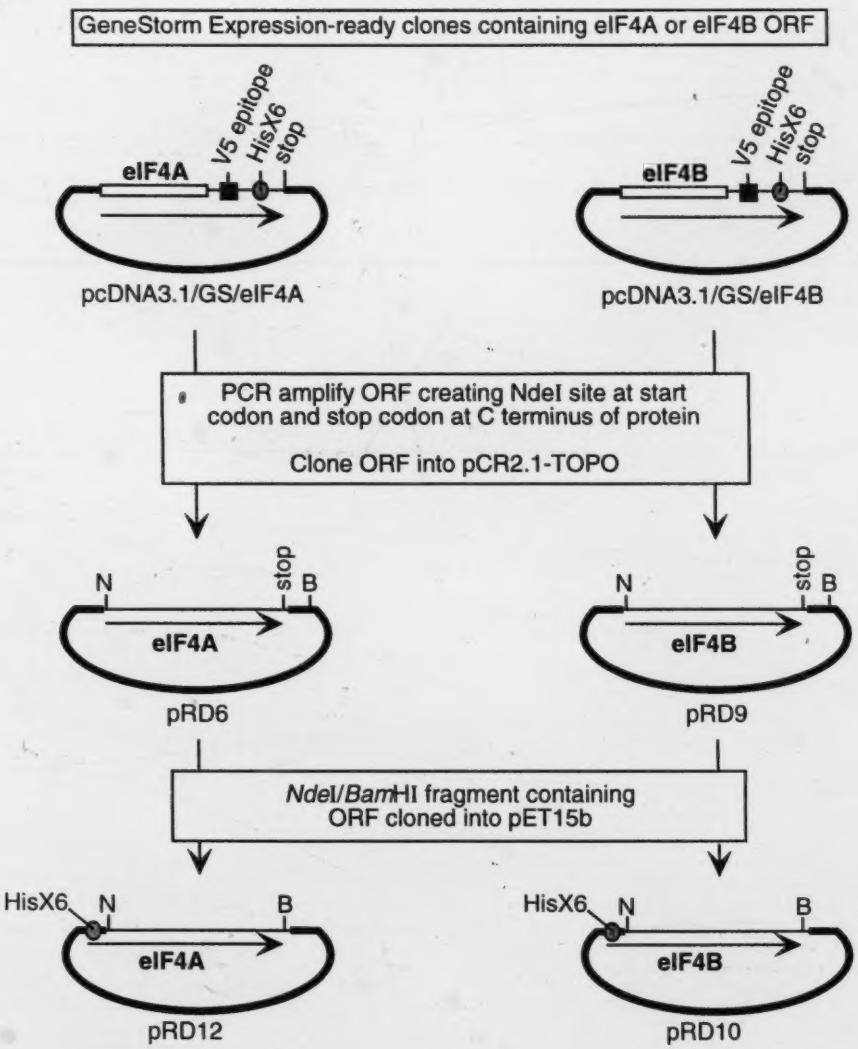

Hela cell RNA
RT PCR to obtain eIF4H ORF
Clone ORF into pGEM-T easy
CCCCCC
S N
X N Sa
eIF4H
pSW1
CCCCCCC
S X
X
N Sa
eIF4Hi
pSW2
SphI/ SacI fragment cloned into pSelect-1
Oligo-directed mutagenesis
SphI/NdeI fragment cloned into pET15b
CCCCCCC
S N
X N Sa
eIF4H
pGC2
HisX6
N
X
X B
eIF4Hi
pRD2
NdeI fragment cloned into pET15b
HisX6
N
X N X B
eIF4H
pRD1

Figure 2.2: Diagram of the cloning strategy to create the eIF4A and eIF4B expression vectors. The eIF4A ORF was cloned into pCR2.1-TOPO to generate pRD6 and finally into the expression vector pET15b to create pRD12. The eIF4B ORF was cloned into pCR2.1-TOPO to generate pRD9 and then into the expression vector pET15b to produce pRD10. See Materials and Methods for more details. Restriction sites are as follows: N - *Nde*I, and B – *Bam*HI.


GeneStorm Expression-ready clones containing eIF4A or eIF4B ORF
V5 epitope
HisX6
stop
eIF4A
pcDNA3.1/GS/eIF4A
eIF4B
pcDNA3.1/GS/eIF4B
PCR amplify ORF creating NdeI site at start codon and stop codon at C terminus of protein
Clone ORF into pCR2.1-TOPO
N
stop
B
eIF4A
pRD6
eIF4B
pRD9
NdeI/BamHI fragment containing ORF cloned into pET15b
HisX6
pRD12
pRD10

CHAPTER 3 : RESULTS

3.1 EXPRESSION OF VHS IN *S. CEREVISIAE* INDUCES A RIBONUCLEASE ACTIVITY THAT IS STIMULATED BY ONE OR MORE MAMMALIAN FACTORS

A previous study from this laboratory reported that cell-free extracts prepared from yeast engineered to express vhs display little if any RNase activity when assayed under conditions that readily detect the activity of vhs translated in rabbit reticulocyte lysates (RRL) (Lu *et al.*, 2001a). In contrast, vhs-dependent activity was easily detected when unprogrammed RRL was added to the yeast extracts. These observations suggested that vhs produced in yeast requires one or more mammalian factors for efficient activity *in vitro*. Our more recent trials of this experiment confirm the stimulatory effect of RRL (Figure 3.1). However, in contrast to the previous study, we are now able to reproducibly detect vhs-dependent RNase activity in the yeast extracts in the absence of RRL, as shown in the following representative experiment.

Cultures of *S. cerevisiae* harboring a previously described vhs expression vector (2.1vhs) (Lu *et al.*, 2001a) or empty vector (EV) were induced with copper sulfate, and whole cell extracts were prepared as described in Material and Methods. The extracts were then assayed for vhs-dependent RNase activity in the presence and absence of added RRL, as previously described (Lu *et al.*, 2001a). Two RNA substrates were used for this assay: pCITE-1 RNA, which bears the IRES of EMCV at it's 5' end, and SRPα RNA (Figure 3.1A and B). Internally labeled RNAs generated by *in vitro* transcription were added to the extracts, and samples withdrawn at various times were analyzed by formaldehyde-agarose gel electrophoresis followed by autoradiography (Figure 3.1C and D). As controls, the substrate RNAs were also incubated in RRL containing pre-translated vhs (RRLvhs), and in blank RRL. Previous studies have shown that pCITE-1 RNA is initially cleaved immediately 3' of the IRES in the RRL *in vitro* assay system (Diagrammed in Figure 3.1A), giving rise to 5'

and 3' products of 600- and 1800-nt, respectively (Elgadi & Smiley, 1999). The 600-nt fragment containing the IRES is stable throughout the course of the reaction, while the 1800-nt fragment is subjected to further decay. As shown in Figure 3.1C, we readily detected the stable 600-nt fragment in the reaction with RRL containing pretranslated vhs (RRLvhs); however, we could not unambiguously detect the predicted 1800-nt 3' fragment in this experiment, because the input RNA substrate contained small amounts of an RNA species that migrated at this position in the gel (see Figure 3.6 for clearer evidence for production of the 1800-nt 3' fragment). The pCITE-1 RNA was quite stable in the extract prepared from yeast harboring the empty expression vector, both in the absence and presence of added RRL (Figure 3.1C, EV and EV+RRL). However, it was significantly less stable in the extract prepared from yeast induced to express vhs (Figure 3.1C, 2.1vhs, data quantified in Figure 3.1E). This effect, observed in several independently prepared extracts, indicates that expression of vhs in yeast is associated with enhanced RNase activity in cell-free extracts. The degradation profile observed with the 2.1vhs extracts differed from that observed in RRLvhs, in that substantially reduced levels of 600-nt product were detected and 900- and 1200-nt fragments were also produced. These findings raised the possibility that the vhs-dependent RNase detected in the yeast extracts is not targeted to the RNA substrate in the same fashion as in RRL, a hypothesis that is explored in further detail below (Figure 3.6).

Addition of RRL enhanced the RNase activity of the 2.1vhs extracts, and altered the overall pattern, such that substantial amounts of the 600-nt fragment accumulated. The addition of RRL may enhance the recovery of a stable 600-nt fragment by targeting cleavage to just 3' of the IRES (thus causing the nuclease to bypass the IRES), by providing factors that bind the IRES and protect it from degradation, or by a combination of both mechanisms. Broadly similar results were obtained with SRPα RNA, with the exception that in this case discrete reaction products were not readily detected in any of the reactions (Figure 3.1D, data quantified in Figure 3.1F). Thus, the transcript was significantly less stable

in extracts of yeast expressing vhs than in control extracts, and the activity of these extracts was enhanced by the addition of RRL. It is unclear as to why nuclease activity is now detected in yeast extracts containing vhs, however, it does not appear to be due to a difference in the amount of vhs protein present in the extracts. Both 2.1vhs extracts from this study and a previous one by Lu *et al.* (Lu *et al.*, 2001a) contain similar amounts of vhs protein (Figure 3.2 C). Taken in combination, these results indicate that expression of vhs in yeast induces a novel RNase activity that is enhanced, and perhaps altered, by one or more factors present in RRL.

It also appears that the nuclease activity of vhs in yeast extracts has a slightly different specificity for RNAs than vhs translated in RRL. A previous study found that vhs translated in RRL has a stronger preference for pCITE-1 RNA, as compared to SRPα RNA (Lu *et al.*, 2001b). However, the opposite is true in yeast extracts expressing vhs (Figure 3.2 A, data quantified in Figure 3.2 B). In 2.1vhs yeast extracts, SRPα RNA is degraded at a significantly faster rate than pCITE-1 RNA (Figure 3.2 A and B). In contrast, when RRL is added to 2.1vhs extracts, both RNAs are degraded at an almost identical rate. The significance of this observation is not yet known.

3.2 eIF4H Stimulates the Endoribonuclease Activity of vhs Produced in Yeast

As reviewed in the introduction, the initial cleavage events induced by vhs translated in RRL occur near areas of translation initiation (Elgadi *et al.*, 1999, Elgadi & Smiley, 1999). vhs has been shown to interact with the translation initiation factor eIF4H (Feng *et al.*, 2001) and a complex of vhs/eIF4H has RNase activity (Everly *et al.*, 2002). We therefore sought to determine whether eIF4H is the mammalian factor that stimulates the RNase activity of vhs expressed in yeast and/or serves to target vhs to specific sites on mRNAs. The eIF4H cDNA was cloned into a bacterial expression vector to express eIF4H as

an N-terminally His_6-tagged protein that was partially purified as described in Materials and Methods. Both eIF4H and $eIF4H_i$, an alternatively spliced version of eIF4H which contains an insertion of 20 amino acids after residue 137 (Feng *et al.*, 2001), were used in preliminary assays. We did not detect any differences in the activities of the two proteins, so subsequent experiments were performed using only the shorter version of eIF4H. As a negative control, we also expressed a His_6-tagged version of the yeast ubiquitin-conjugating enzyme $cdc34\Delta_{209}$, which is truncated at amino acid 209 (Ptak *et al.*, 1994), a presumably irrelevant protein. Translation initiation factors eIF4A and eIF4B were also expressed and partially purified as described in Material and Methods and were used in later experiments (see Figures 3.8 - 3.12). Figure 3.3 shows a coomassie-stained gel, and western blot analysis of the partially purified proteins. At least some of the extra bands seen below the intact eIF4H and eIF4B on the coomassie-stained gel are degradation products that contain the amino-terminus, as an antibody directed against the His_6-tag reacted with the majority of the degradation products (Figure 3.3 C).

All of the recombinant protein preparations described above (eIF4A, eIF4B, eIF4H and $cdc34\Delta_{209}$) contained small amounts of contaminating RNase activity that was readily detected when the partially purified protein was incubated alone with radiolabeled substrate (although the amount of nuclease activity varied between preparations) (Figure 3.4 C). However, the partially purified proteins had no effect on the stability of the RNA substrate when they were added to control yeast extract (Figure 3.5 and 3.9, EV+eIF4H, EV+eIF4A, EV+eIF4B). This observation suggested that the large excess of yeast protein or (more likely) RNA present in the control extracts served to quench this nuclease activity. Consistent with this interpretation, when eIF4H and $cdc34\Delta_{209}$ were mixed with RNase-free BSA and total RNA purified from control extracts to the same concentration as in control extracts, no appreciable nuclease activity was observed (Figure 3.4 A and B, eIF4H and $cdc34\Delta_{209}$). We therefore judged that

these preparations were suitable for testing the effects of the various partially purified proteins on the vhs-dependent nuclease in yeast extracts.

Partially purified eIF4H was used to investigate whether eIF4H affects the activity of vhs produced in yeast. As described above, eIF4H had no detectable effect on the stability of the RNA substrates in extracts prepared from yeast harboring the empty expression vector (Figure 3.5 A and B, EV+eIF4H). In marked contrast, addition of eIF4H to the 2.1vhs extracts significantly accelerated the decay of both pCITE-1 and SRPα RNAs (Figure 3.5 A and B, 2.1vhs+eIF4H, data quantified in Figure 3.5 C and D). This effect appears to be specific, as a presumably irrelevant protein (yeast cdc34Δ_{209}), purified in the same manner as eIF4H, had no such effect (Figure 3.4 A and B, 2.1vhs+cdc34). In addition, eIF4H altered the pattern of degradation products of pCITE-1 RNA compared to that observed in 2.1vhs extracts alone. Specifically, more of the 600-, 900- and 1200-nt degradation products were produced, and the 600-nt fragment was quite stable even after the disappearance of the 900- and 1200-nt fragments (Figure 3.5 A, 2.1vhs+eIF4H). The 600-nt product co-migrated with the 600-nt 5' fragment produced in RRLvhs, and as documented below, at least some of the material migrating at this position arises by cleavage just downstream of the IRES (Figure 3.6). While the predicted 1800-nt 3' fragment diagnostic of IRES targeting was not observed with eIF4H, the detection of this fragment may have been compromised because the input RNA contained RNA species that migrated at the same position (as in Figure 3.1). Although eIF4H altered the pattern of degradation products, it did not have the same effect as adding RRL to the 2.1vhs extracts. With the addition of RRL, the degradation product profile was much more similar to that observed in the intact RRL assay, in that substantial amounts of the 600- and 1800-nt fragments and much less (if any) of the 900- and 1200-nt fragments were produced. Thus, although eIF4H accelerated the cleavage of the RNA substrates, these data raise the possibility that it does not efficiently reconstitute preferential cleavage to downstream of the EMCV IRES. This point is examined further below (Figure 3.6).

3.3 Effects of RRL and eIF4H on Targeting of vhs Activity to the EMCV IRES.

Elgadi and Smiley provided evidence that the EMCV IRES strongly targets the initial vhs-induced cleavage events to sequences located just downstream of the IRES in the RRL assay system (Elgadi & Smiley, 1999). Two of the key observations that led to this conclusion were as follows. First, when internally labeled pCITE-1 RNA was used as the substrate, 600-nt and 1800-nt degradation products were simultaneously produced early during the reaction, with no evidence of prior intermediates (Elgadi & Smiley, 1999). These two products roughly add up to equal the length of the intact substrate. Second, the 600- and 1800-nt fragments were shown to correspond to the 5' and 3' ends of the transcript respectively, using direct and indirect end-labeling techniques (Elgadi & Smiley, 1999). The experiments depicted in Figures 3.1 and 3.5 did not provide definitive information about the targeting properties of vhs expressed in yeast (either in the presence or absence of mammalian factors) because we could not unambiguously detect the 1800-nt 3' degradation fragment, and the origin of the various degradation intermediates was not examined in detail.

To more rigorously determine if degradation of pCITE-1 RNA proceeds in the same manner as in the intact RRL system in 2.1vhs extracts following reconstitution with RRL or eIF4H, we first repeated the experiment using internally labeled substrate. The experiment was modified in two ways to maximize the likelihood of detecting the unstable 1800 nt 3' fragment: a more homogenous substrate was used, and samples were taken at earlier time points (Figure 3.6 A). The degradation pattern observed in the 2.1vhs extract supplemented with RRL closely resembled that previously observed in the intact RRL system (Elgadi & Smiley, 1999), in that 1800- and 600-nt products were clearly evident at early times, and these were the only obvious discrete

degradation fragments detected (Figure 3.6 A, 2.1vhs+RRL). Moreover, the 600-nt fragment was quite stable and accumulated throughout the reaction while the 1800-nt fragment was subject to further decay. However, this was not the case in reactions supplemented with eIF4H. By 1.5 min, possibly up to four degradation products could be detected (Figure 3.6 A, 2.1vhs+eIF4H). Thus, 600-, 900-, 1200- and possibly 1800-nt fragments were produced, although the 1800-nt fragment was not one of the most predominant and was rapidly degraded. The 900- and 1200-nt fragments were initially the most predominant but were subject to further decay, while the 600-nt fragment accumulated at later time points. These observations suggest that in the presence of eIF4H, vhs activity is not as strongly targeted by the EMCV IRES as in the presence of RRL.

We next used a slightly modified version of the vhs activity assay to track the fate of the 5' and 3' ends of the RNA substrate over the course of the reaction. Unlabeled pCITE-1 RNA was incubated with variously supplemented extracts, followed by northern blot analysis using oligonucleotide probes complementary to the extreme 5' and 3' ends of the RNA (Figure 3.6 B). As previously described (Elgadi & Smiley, 1999), the earliest products detected in RRLvhs were a stable 5' 600-nt fragment and a predominant 3' 1800-nt fragment (Figure 3.6 C and D, RRLvhs). The 600-nt fragment was essentially the only product detected with the 5' probe, and it arises through cleavage just downstream of the IRES. While the 1800-nt fragment was the predominant 3' product detected at early times, it was not the only 3' degradation product present. Over the time course, the 1800-nt fragment was subject to further decay and a shift from larger to smaller products occurred, with discrete intermediates. The sizes of these further degradation products map the positions of preferential cleavage sites, from 5' to 3', as the initial 3' product is further degraded. The overall pattern of degradation products observed in RRLvhs is consistent with a model where vhs cleaves the RNA in an overall 5' to 3' direction. Indeed, additional experiments using oligonucleotides

complementary to multiple sites along the length of the RNA confirm this interpretation (Perez-Prada and Smiley, unpublished results).

The 2.1vhs extracts showed a comparable loss of intact substrate, but comparably abundant discrete degradation products were not detected (Figure 3.6 C and D, 2.1vhs). We have reproductively seen faint bands that co-migrate with some the degradation products in RRLvhs, however at a much lower molar yield. These observations demonstrate that 2.1vhs is not strongly targeted by the EMCV IRES. In contrast, RRL altered the pattern of degradation products to one almost identical to that seen with RRLvhs. A dominant 5' 600-nt fragment containing the IRES was detected, although faint larger 5' fragments were also observed. Furthermore, the 3' 1800-nt fragment was the most abundant product at early times, although other smaller 3' products were also seen. However, unlike in RRLvhs, the 3' degradation products were not obviously shifted from larger to smaller fragments over the course of the reaction. Further testing is required to determine if vhs cleaves the RNA in a 5' to 3' direction in yeast extracts supplemented with RRL. eIF4H also altered the degradation profile of the 2.1vhs extracts, however, the pattern was quite different from that seen with RRL (Figure 3.6 C and D, 2.1vhs+eIF4H). The 600-nt 5' fragment bearing the IRES was detected, however the molar yield was substantially lower and additional larger 5' products were also produced at early times. Furthermore, the 3' 1800-nt fragment was barely detectable; rather, smaller 3' products were more abundant. These observations indicate that in the presence of eIF4H, the sites of initial cleavage of pCITE-1 RNA are distributed throughout the length of the RNA, rather than focused immediately downstream of the IRES as in reactions containing RRL. Taken in combination, these results demonstrate that although RRL restores IRES-mediated targeting to vhs expressed in yeast, eIF4H does not.

3.4 eIF4B Interacts with vhs

We decided to examine two other translation initiation factors for their ability to interact with and stimulate the RNase activity of vhs produced in yeast. The first translation initiation factor chosen was eIF4B, which is a sequence paralogue of eIF4H. These two proteins work together to modulate the helicase activity of eIF4A (Richter *et al.*, 1999, Rogers *et al.*, 1999, Rogers *et al.*, 2001). They have 62% similarity and 39% identity in their amino acid sequence (Richter-Cook *et al.*, 1998). By using the Blast 2 sequences alignment program (see Figure 3.7 for parameters) (Tatusova & Madden, 1999), the region of greatest similarity was found between residues 33-117 of eIF4H and 87-147 of eIF4B (Figure 3.7). This region of strong similarity overlaps with the region of eIF4H that interacts with vhs (residues 90-137) (Feng *et al.*, 2001), raising the possibility that eIF4B may also interact with vhs. The second candidate protein we investigated was eIF4A. eIF4A is an RNA helicase which is a component of eIF4F, a translation initiation factor that binds the cap of mRNAs, and is known to interact functionally with both eIF4H and eIF4B in translation initiation (Richter *et al.*, 1999, Rogers *et al.*, 1999, Rogers *et al.*, 2001). Both eIF4A and eIF4B were expressed as His_6-tagged proteins and purified in exactly the same manner as eIF4H (Figure 3.3, eIF4A and eIF4B).. The eIF4B preparation contained both the full-length protein as well as a series of smaller polypeptides, some of which were quite abundant. The smaller species are presumably degradation products of eIF4B as an anti-His-tag antibody reacted with some of the polypeptides (Figure 3.3 B). The degradation of eIF4B in *E. coli* has been previously noted by Methot *et al.* (Methot *et al.*, 1994), who also reported that the degradation products do not interfere with the ability of eIF4B to stimulate the helicase activity of eIF4A. To compensate for the presence of these degradation products we used equimolar amounts of full-length proteins in the assays described below.

Potential interactions between vhs and eIF4B and eIF4A were analyzed by far western blot analysis using radio-labeled vhs as a probe. Partially purified translation factors were resolved on a SDS-PAGE gel, transferred to a

nitrocellulose membrane and probed with RRL containing ^{35}S-labeled vhs. eIF4H acted as a positive control as it has already been shown to interact with vhs (Feng *et al.*, 2001). cdc34Δ_{209}, BSA and protein phosphatase 1were used as negative-control proteins and an interaction with vhs was not detected (Figure 3.8 A and B, cdc34, BSA, PP1). eIF4H and eIF4B were both found to interact with vhs, as seen by the appearance of bands on the far western blot corresponding to the positions of the proteins on the coomassie-stained gel (Figure 3.8 A and B, eIF4H and eIF4B). While the signal intensity obtained with eIF4B was lower than with eIF4H, it is unclear whether this is due to a lower affinity for vhs or the consequence of a lower quantity of intact eIF4B compared to eIF4H present on the blot. An interaction between vhs and eIF4A was not detected (Figure 3.8 A and B, eIF4A). However, while we did not observe an interaction between eIF4A and vhs, the possibility of detecting an interaction by other methods cannot be ruled out. These data show that both eIF4H and eIF4B can directly interact with vhs *in vitro*.

3.5 eIF4B Stimulates the Endoribonuclease Activity of vhs Expressed in Yeast

We next investigated whether eIF4B or eIF4A could stimulate the RNase activity or reconstitute IRES-directed targeting to vhs produced in yeast. Vhs activity assays were performed as described for Figure 3.6 adding either eIF4A or eIF4B to yeast extracts. The concentration of eIF4H and eIF4B used in these assays was 2.1μM, as this was the highest concentration of full-length eIF4B that we were able to achieve. Both pCITE-1 and SRPα RNA substrates were very stable in control extracts in the presence of eIF4A and eIF4B (Figure 3.9 A and B, EV+eIF4A, EV+eIF4B). eIF4B enhanced the RNase activity of vhs on pCITE-1 RNA and the degradation product profile was similar to that seen when eIF4H is added to 2.1vhs extracts, with the exception that the 600 nt product was perhaps less stable (Figure 3.9 A, 2.1vhs+eIF4B, data quantified in Figure

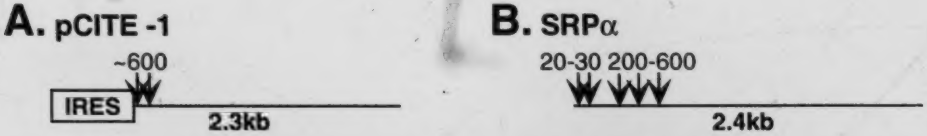
3.9 C). Similar results were seen with SRPα RNA, with the exception that discrete degradation products were not observed (Figure 3.9 B, 2.1vhs+eIF4B, data quantified in figure 3.9 D). The ability of eIF4B to enhance the activity of vhs produced in yeast may vary with the substrate used, as eIF4B did not enhance the decay of pCITE-1 RNA as much as SRPα RNA, relative to the effect of eIF4H (Figure 3.9 C versus D, 2.1vhs+eIF4B).

While eIF4A did not affect the nuclease activity of the 2.1vhs extracts on pCITE-1 RNA, a slight stimulation was observed with SRPα RNA (Figure 3.9 B, 2.1vhs+eIF4A, data quantified in Figure 3.9 D). The concentration of eIF4A used in these experiments was 7.6 μM to match that of eIF4H used in previous figures. However, when the concentration of eIF4A was lowered to 2.1 μM to match that of eIF4H and eIF4B used in these particular experiments, an enhancement of nuclease activity of vhs could not be detected (Figure 3.10). The purified eIF4A protein retained RNA helicase activity as seen by the increase in ssRNA over time in the presence of eIF4A and ATP (Figure 3.11 A, data quantified in Figure 3.11 B), suggesting that the protein preparation was functional. Thus, the inability of eIF4A to enhance the nuclease activity of vhs produced in yeast was not due to a non-functional eIF4A protein. It is important to note that, while the eIF4A and eIF4B protein preparation contained similar amounts of contaminating nuclease activity (Figure 3.4 C), the same amount of each protein added to the 2.1vhs extracts had very different effects (Figure 3.9 vs 3.10). eIF4B enhanced the nuclease activity of the 2.1vhs extracts, where as eIF4A did not have such an effect. Thus, the enhancement of the nuclease activity of 2.1vhs is most likely not due to contaminating bacterial nucleases.

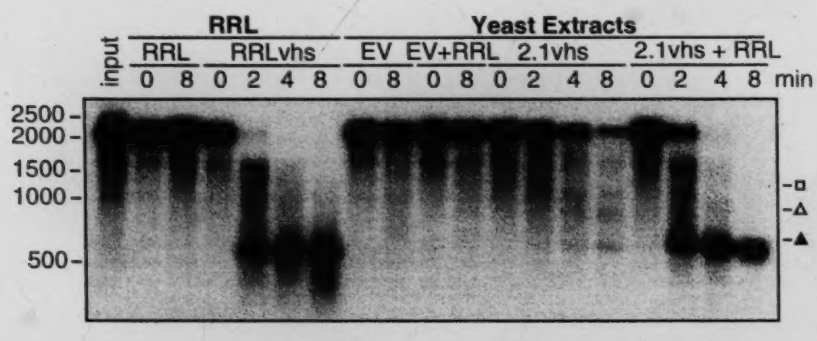
We next used the modified vhs activity assay to monitor the fate of the 5' and 3' ends of pCITE-1 RNA when eIF4A and eIF4B were added to 2.1vhs extracts (Figure 3.12). As was the case for eIF4H, with both eIF4A and eIF4B the 3' 1800-nt fragment was almost undetectable (Figure 3.12 B, +eIF4A, +eIF4B). Additionally, the 5' 600-nt IRES-containing fragment was almost nonexistent with eIF4A and the molar yield was substantially lower with eIF4B

than with RRL (Figure 3.12 A, +eIF4A, +eIF4B). These observations indicate that eIF4A and eIF4B are unable to restore IRES-directed targeting to 2.1vhs. In addition, eIF4H and eIF4A in combination and eIF4B and eIF4H in combination were also unable to reconstitute targeting to 2.1vhs, as seen by the lack of detection of a strong 3' 1800-nt fragment with the corresponding 5' 600-nt fragment (Figure 3.12 A and B). Taken together, these results demonstrate that while eIF4B and eIF4H stimulate the activity of 2.1vhs, one or more other additional mammalian factors are required to fully reconstitute IRES-mediated targeting to 2.1vhs.

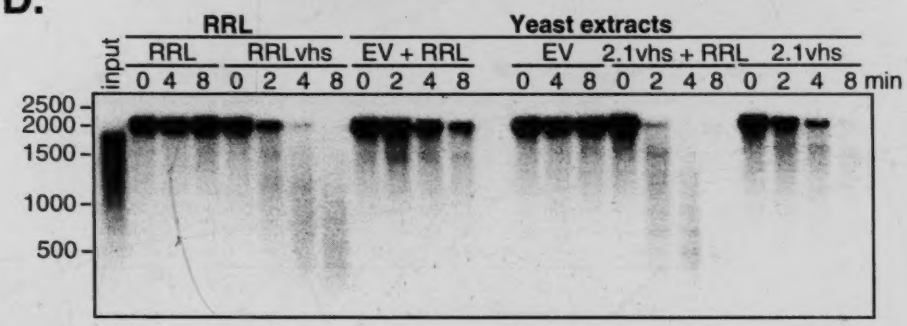
Figure 3.1: Extracts of yeast expressing 2.1vhs contain endoribonuclease activity that is enhanced by one or more mammalian cofactors. (A and B) Diagrams of pCITE-1 and SRPα RNA substrates, respectively, showing the sites of initial cleavage events by vhs translated in RRL. The EMCV IRES on pCITE-1 is indicated. (C and D) Analysis of nuclease activity on pCITE-1 and SRPα RNA substrates, respectively. Internally labeled pCITE-1 and SRPα RNA was added to control RRL (RRL), RRL pretranslated with vhs (RRLvhs), extracts of yeast harboring the empty expression vector either alone (EV) or mixed with RRL (EV+RRL), and extracts of yeast expressing 2.1vhs either alone (2.1vhs) or mixed with RRL (2.1vhs+RRL). RNA was extracted at the indicated time points, resolved on a 1.2 % agarose – 2 % formaldehyde gel, transferred to a Gene Screen Plus membrane, and the RNA signal was detected by autoradiography(panels C and D). The solid triangle indicates the previously described 600-nt degradation product corresponding to the EMCV IRES. The open square and triangle correspond to the 1200- and 900-nt degradation fragments. The position and size of the markers is indicated in nucleotides at the left. (E and F) Quantification of nuclease activity on pCITE-1 and SRPα RNAs, respectively. The quantity of full-length RNA from three experiments were determined using phosphor-imager analysis and plotted against the time points indicated. The error bars indicate the standard deviation of each time point calculated from at least three independent experiments.



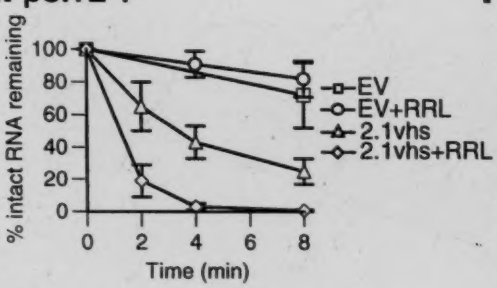
C.



D.



E. pCITE-1



F. SRPα

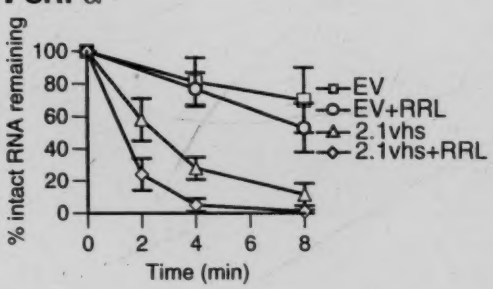


Figure 3.2: Comparison of the nuclease activity of 2.1vhs yeast extracts on pCITE-1 and SRPα RNA substrates. (A) Analysis of nuclease activity using pCITE-1 and SRPα RNA substrates. Internally labeled pCITE-1 and SRPα RNA was added to control RRL (RRL), and extracts of yeast expressing 2.1vhs either alone (2.1vhs) or mixed with RRL (2.1vhs+RRL). Samples were processed as described in the legend of Figure 3.1. The solid triangle indicates the previously described 600-nt degradation product corresponding to the EMCV IRES. The position and size of the markers is indicated in nucleotides at the left. (B) Quantification of nuclease activity on pCITE-1 and SRPα RNAs. The quantity of full-length RNA from three experiments were determined using phosphor-imager analysis and plotted against the time points indicated. The error bars indicate the standard deviation of each time point calculated from at least three independent experiments. (C) Western blot analysis of yeast extracts. Yeast extracts prepared for this study were analyzed by western blot and compared to those prepared for a previous study by Lu *et al.* (Lu *et al.*, 2001a). Yeast extracts containing the empty vector and expressing vhs were run on a 12% SDS-PAGE gel, transferred to nitrocellulose membrane and the vhs protein was detected using a monoclonal antibody directed against the HA epitope.

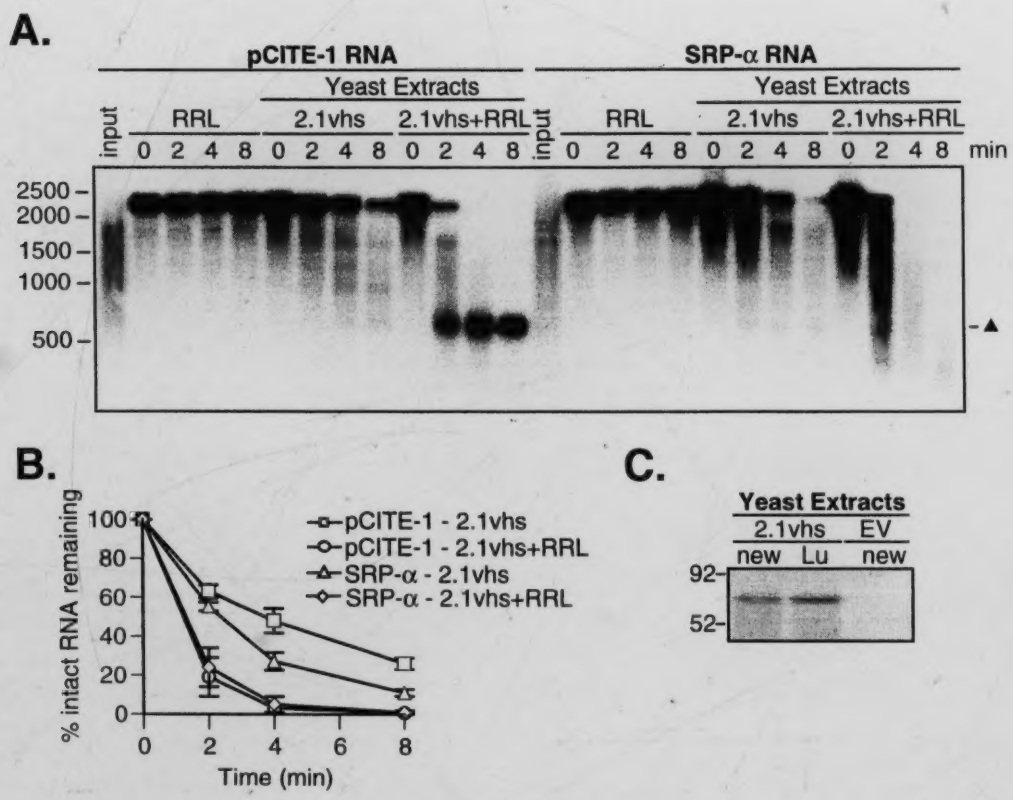
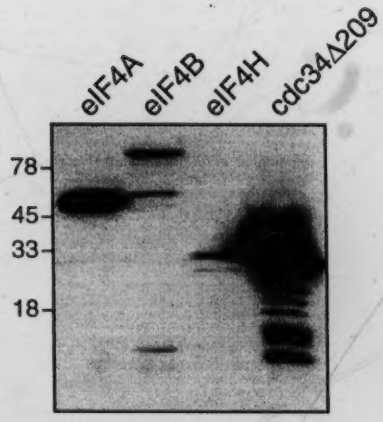

A.
pCITE-1 RNA
SRP-α RNA
Yeast Extracts
input
RRL
2.1vhs
2.1vhs+RRL
0 2 4 8
min
2500
2000
1500
1000
500
B.
% intact RNA remaining
Time (min)
pCITE-1 - 2.1vhs
pCITE-1 - 2.1vhs+RRL
SRP-α - 2.1vhs
SRP-α - 2.1vhs+RRL
C.
Yeast Extracts
2.1vhs
EV
new
Lu
92
52

Figure 3.3: Analysis of purified proteins. (A) Partially purified eIF4H, eIF4A, eIF4B and cdc34Δ_{209} (5μg of each) were resolved on a 12 % SDS-PAGE gel and stained with coomassie brilliant blue. (B and C) Western blot analysis of partially purified proteins. EIF4H, eIF4A, eIF4B and cdc34Δ_{209} (1 μg of full length protein, panel B, and 4 μg of eIF4H, panel C) were resolved on a 12.5 % SDS PAGE gel, transferred to a nitrocellulose membrane and proteins were detected using the anti-Tetra-His antibody (Quiagen). The position and size of the protein markers are indicated in kilodaltons on the left.

A.

eIF4H
eIF4A
eIF4B
cdc34Δ209
97
66
45
31
21
14

B.


eIF4A
eIF4B
eIF4H
cdc34Δ209
78
45
33
18

C.

eIF4H
29
20

Figure 3.4: The enhancement of the RNase activity of vhs expressed in yeast is specific to eIF4H. (A and B) Analysis of nuclease activity on pCITE-1 and SRPα RNAs, respectively. Internally labeled pCITE-1 and SRPα RNAs were added to yeast extracts containing 2.1vhs either alone, mixed with eIF4H (+eIF4H) or mixed with cdc34Δ_{209} (+cdc34). The RNA substrates were also added to eIF4H or cdc34Δ_{209} mixed with 20 μg RNase-free BSA (Ambion) and 1μg total yeast RNA (extracted from empty vector yeast extracts) per time point. eIF4H and cdc34Δ_{209} were used at a concentration of 7.7 μM. (C) Analysis of nuclease activity on pCITE-1 RNA. Internally labeled pCITE-1 RNA was added to protein preparations of eIF4A, eIF4B, eIF4H and cdc34Δ_{209}. No additional RNA or protein was added to the preparations. Samples were processed as described in the legend of Figure 3.1. The position and size of the RNA markers in nucleotides are indicated at the left.

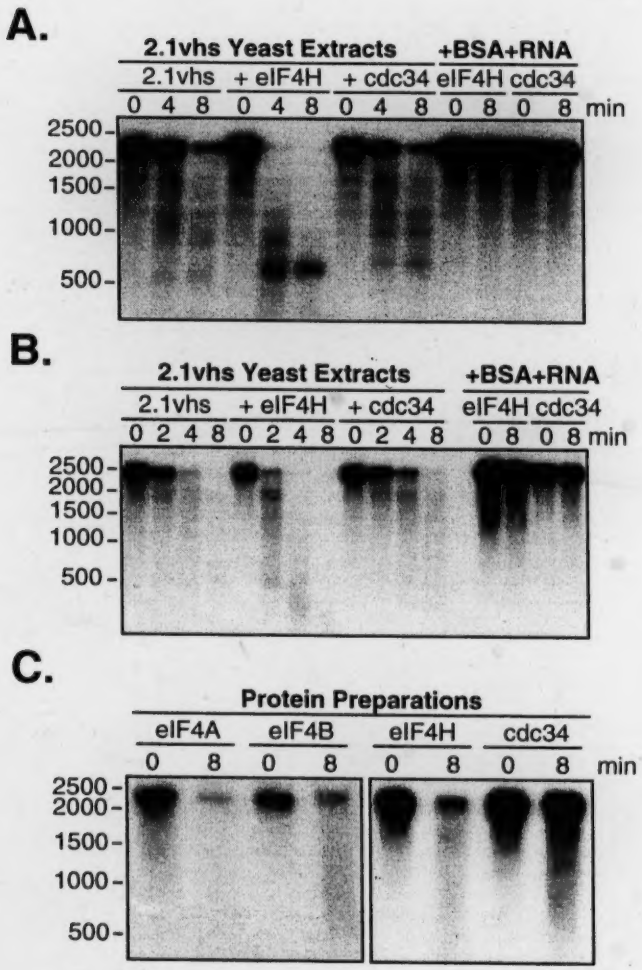

A.
2.1vhs Yeast Extracts
+BSA+RNA
2.1vhs
+ eIF4H
+ cdc34
eIF4H
cdc34
0 4 8 0 4 8 0 4 8 0 8 0 8 min
2500
2000
1500
1000
500
B.
2.1vhs Yeast Extracts
+BSA+RNA
2.1vhs
+ eIF4H
+ cdc34
eIF4H
cdc34
0 2 4 8 0 2 4 8 0 2 4 8 0 8 0 8 min
2500
2000
1500
1000
500
C.
Protein Preparations
eIF4A
eIF4B
eIF4H
cdc34
0 8 0 8 0 8 0 8 min
2500
2000
1500
1000
500

Figure 3.5: eIF4H stimulates the endoribonuclease activity of vhs produced in yeast. (A and B) Analysis of nuclease activity on pCITE-1 and SRPα RNA substrates, respectively. Internally labeled pCITE-1 and SRPα RNAs were added to control RRL (RRL), eIF4H mixed with vhs assay buffer (eIF4H), control extracts: alone (EV), mixed with RRL (+RRL), or mixed with eIF4H (+eIF4H) and yeast extracts containing 2.1vhs: alone (2.1vhs), mixed with RRL (+RRL) or mixed with eIF4H (+eIF4H). eIF4H was used at a concentration of 7.7μM. Samples were processed as described in the legend of Figure 3.1. The solid triangle indicates the 600-nt fragment. The open square and triangles indicate additional ca. 1200- and 900-nt degradation products, respectively. The position and size of the RNA markers are indicated in nucleotides at the left. (C and D) Quantification of nuclease activity on pCITE-1 and SRPα RNAs, respectively. The quantity of full-length RNA from three experiments was determined using phosphor-imager analysis and plotted against time points indicated. The error bars indicate the standard deviation of each time point calculated from at least three independent experiments.

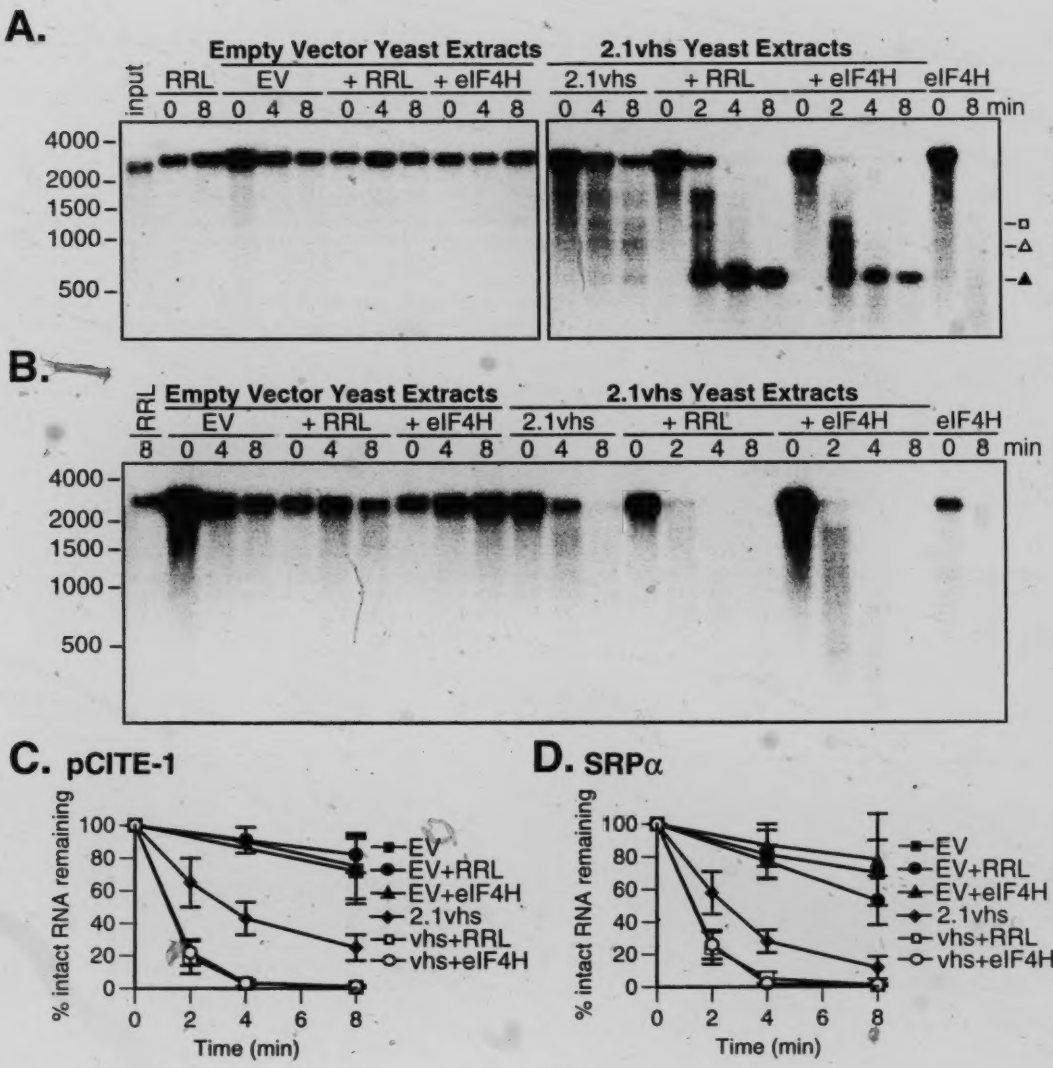

A.
Empty Vector Yeast Extracts
2.1vhs Yeast Extracts
input
RRL
EV
+ RRL
+ eIF4H
2.1vhs
eIF4H
0 8 0 4 8 0 4 8 0 4 8 0 4 8 0 2 4 8 0 2 4 8 0 8 min
4000
2000
1500
1000
500
B.
RRL
Empty Vector Yeast Extracts
2.1vhs Yeast Extracts
EV
+ RRL
+ eIF4H
2.1vhs
eIF4H
8 0 4 8 0 4 8 0 4 8 0 4 8 0 2 4 8 0 2 4 8 0 8 min
4000
2000
1500
1000
500
C. pCITE-1
% intact RNA remaining
Time (min)
EV
EV+RRL
EV+eIF4H
2.1vhs
vhs+RRL
vhs+eIF4H
D. SRPα
% intact RNA remaining
Time (min)
EV
EV+RRL
EV+eIF4H
2.1vhs
vhs+RRL
vhs+eIF4H

Figure 3.6: eIF4H does not restore efficient IRES-directed targeting to yeast-expressed vhs. (A) Analysis of nuclease activity on pCITE-1 RNA. Internally labeled pCITE-1 RNA was added to control RRL (RRL), and yeast extracts containing 2.1vhs in the presence and absence of RRL or eIF4H (2.1vhs, 2.1vhs+RRL, 2.1vhs+eIF4H). Samples were processed as described in the legend of Figure 3.1. (B) Diagram of pCITE-1 RNA, indicating the positions of hybridization of oligonucleotides ORD17 and JPP-E specific for the 5' and 3' ends of the RNA, respectively. Also indicated are the RNA fragments produced upon initial cleavage of the RNA by vhs translated in RRL. (C and D) Northern blot analysis of nuclease activity on pCITE-1 RNA using oligonucleotide probes for the 5' and 3' ends of pCITE-1 RNA, respectively. Unlabeled pCITE-1 RNA was added to control RRL(RRL), RRL containing pretranslated vhs (RRLvhs), and yeast extracts containing 2.1vhs, either alone (2.1vhs), mixed with RRL(2.1vhs+RRL) or mixed with eIF4H(2.1vhs+eIF4H). RNA was extracted at the indicated time points, resolved on an agarose-formaldyhyde gel, transferred to a Gene Screen Plus membrane and analyzed by Northern blot analysis. Symbols are as described in the legend of Figure 3.1, the closed square indicates the position of the 1800-nt fragment. The position and size of the RNA markers in nucleotides are at the left.

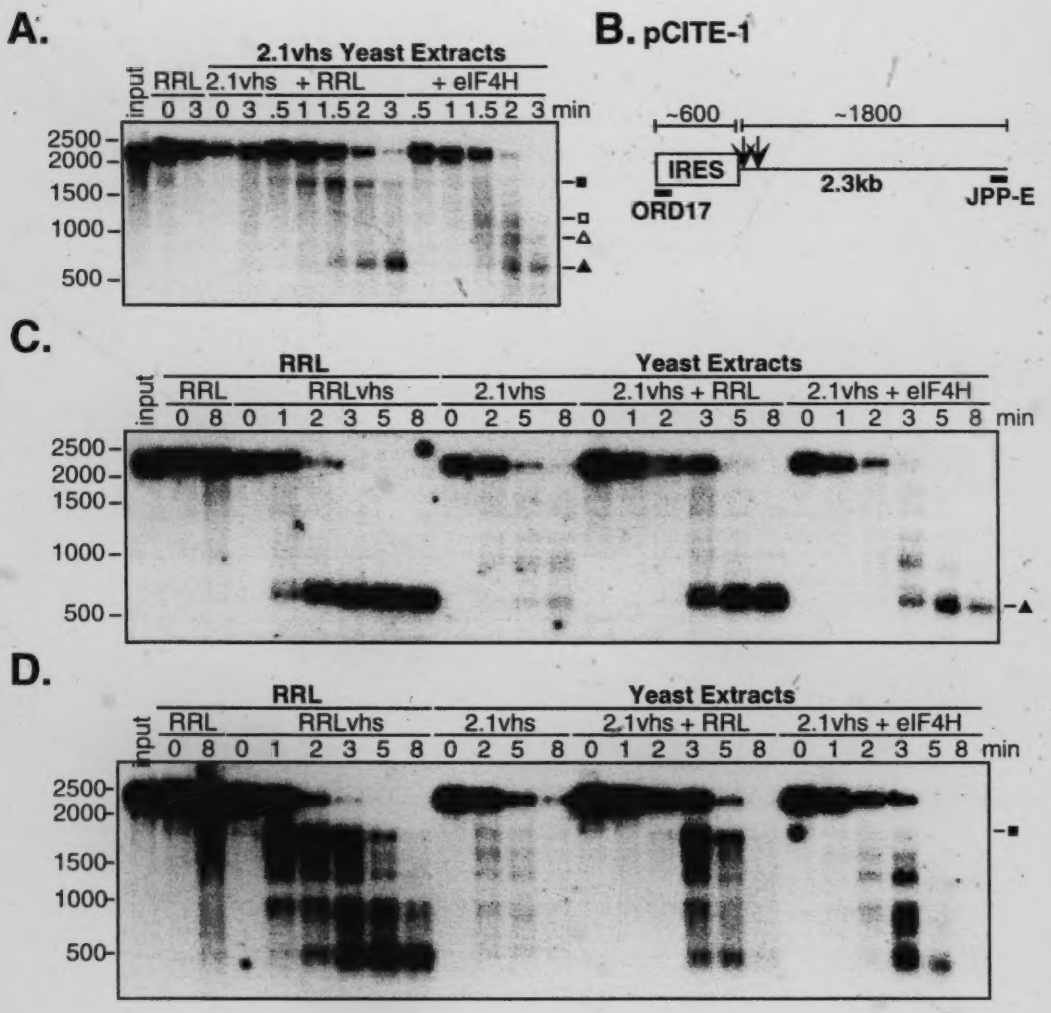

A.
2.1vhs Yeast Extracts
input
RRL
2.1vhs
+ RRL
+ eIF4H
0 3 0 3 .5 1 1.5 2 3 .5 1 1.5 2 3 min
2500
2000
1500
1000
500
B. pCITE-1
~600
~1800
IRES
2.3kb
ORD17
JPP-E
C.
RRL
Yeast Extracts
input
RRL
RRLvhs
2.1vhs
2.1vhs + RRL
2.1vhs + eIF4H
0 8 0 1 2 3 5 8 0 2 5 8 0 1 2 3 5 8 0 1 2 3 5 8 min
2500
2000
1500
1000
500
D.
RRL
Yeast Extracts
input
RRL
RRLvhs
2.1vhs
2.1vhs + RRL
2.1vhs + eIF4H
0 8 0 1 2 3 5 8 0 2 5 8 0 1 2 3 5 8 0 1 2 3 5 8 min
2500
2000
1500
1000
500

Figure 3.7: Region of greatest similarity between eIF4H and eIF4B. Sequence similarity between residues 33-117 of eIF4H (accession no. NP_114381) and 87-147 of eIF4B (accession no. CAA39265) was determined using the Blast 2 sequences alignment program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) (Tatusova & Madden, 1999). The parameters were as follows: program: blastp, matrix: BLOSUM62, gap penalties: open gap 11, and extension gap 1. The residues shown between the two sequences are conserved and the residues from the same family are indicated as a "+". There is 43 % identity and 68 % similarity between eIF4H and eIF4B in this region.

```
eIF4H: 33  QKELPTEPPYTAYVGNLPFNTVQGDIDAIFKDLSIRSVRLVRD-KDTDKFKGFCYV 87
           +  LP  PPYTA++GNLP++  +  I   F+ L+I +VRL R+  + ++ KGF Y
eIF4B: 87  RSRLPKSPPYTAFLGNLPYDVTEESIKEFFRGLNISAVRLPREPSNPERLKGFGYA 142

eIF4H: 88  EFDEVDSLKEALTYDGALLGDRSLRVDIAE 117
           EF+++DSL  AL+  +  LG+R +RVD+A+
eIF4B: 143 EFEDLDSLLSALSLNEESLGNRRIRVDVAD 172
```

Figure 3.8: eIF4B interacts *in vitro* with vhs. (A) Far western analysis of interactions between vhs and the proteins indicated. 8µg of each protein was resolved on a 12 % SDS-PAGE gel, transferred to a nitrocellulose membrane, incubated with RRL containing pretranslated ^{35}S-labeled vhs and the interaction was detected by autoradiography. (B) Analysis of purified proteins. After transfer of proteins to the membrane, the SDS-PAGE gel was stained for residual protein with coomassie brilliant blue. The position and size of the protein markers in kilodaltons is indicated on the left.

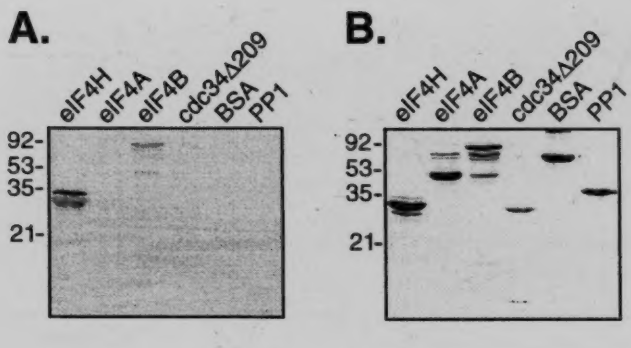
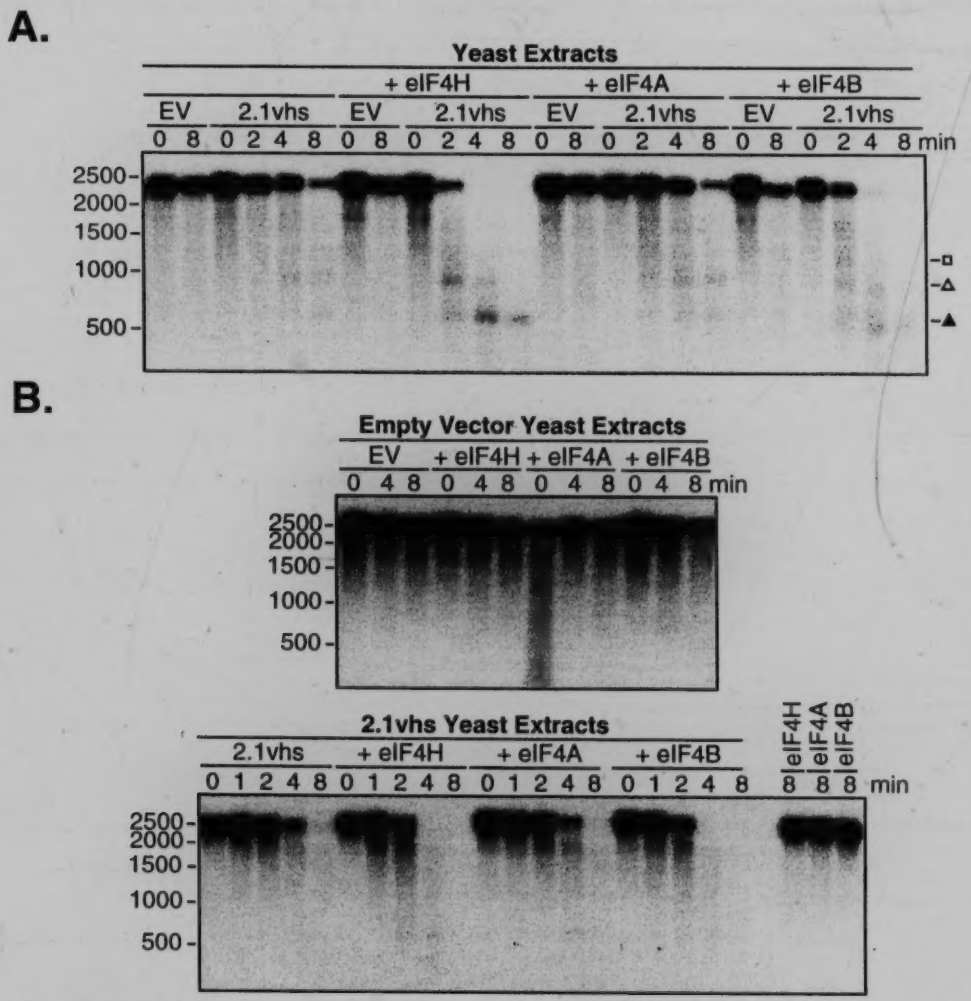
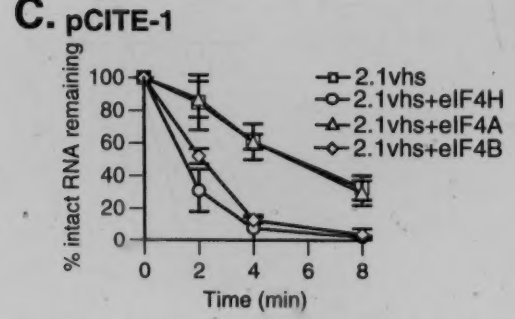

A.
eIF4H
eIF4A
eIF4B
cdc34Δ209
BSA
PP1
92-
53-
35-
21-
B.
eIF4H
eIF4A
eIF4B
cdc34Δ209
BSA
PP1
92-
53-
35-
21-

Figure 3.9: eIF4B stimulates the endoribonuclease activity of the vhs protein produced in yeast. (A and B) Analysis of nuclease activity on pCITE-1 and SRPα RNAs, respectively. Internally labeled pCITE-1 and SRPα RNAs were added to extracts of yeast containing the empty expression vector either alone (EV), mixed with eIF4H (+eIF4H), mixed with eIF4A(+eIF4A) or mixed with eIF4B(+eIF4B), and yeast extracts containing 2.1vhs either alone(2.1vhs), mixed with eIF4H (+eIF4H), mixed with eIF4A(+eIF4A) or mixed with eIF4B(+eIF4B). The concentration of purified proteins used was as follows: eIF4H – 2.1μM, eIF4B – 2.1μM, and eIF4A – 7.6μM. Purified proteins were mixed with Buffer B, and 20μg of RNase-free BSA and 1μg total yeast RNA (extracted from empty vector yeast extracts) per time point. Symbols are as described in the legend of Figure 3.1. The position and size of the RNA markers in nucleotides are at the left. (C and D) Quantification of nuclease activity on pCITE-1 and SRPα RNA substrates, respectively. The quantity of full-length RNA from three experiments were determined using phosphor-imager analysis and plotted against time points indicated. The error bars indicate the standard deviation of each time point calculated from at least three independent experiments.


A.
Yeast Extracts
+ eIF4H
+ eIF4A
+ eIF4B
EV
2.1vhs
0 8 0 2 4 8 0 8 0 2 4 8 0 8 0 2 4 8 0 8 0 2 4 8 min
2500
2000
1500
1000
500
B.
Empty Vector Yeast Extracts
EV
+ eIF4H
+ eIF4A
+ eIF4B
0 4 8 0 4 8 0 4 8 0 4 8 min
2500
2000
1500
1000
500
2.1vhs Yeast Extracts
2.1vhs
+ eIF4H
+ eIF4A
+ eIF4B
0 1 2 4 8 0 1 2 4 8 0 1 2 4 8 0 1 2 4 8
eIF4H
eIF4A
eIF4B
8 8 8 min
2500
2000
1500
1000
500


C. pCITE-1
% intact RNA remaining
100
80
60
40
20
0
0 2 4 6 8
Time (min)
2.1vhs
2.1vhs+eIF4H
2.1vhs+eIF4A
2.1vhs+eIF4B

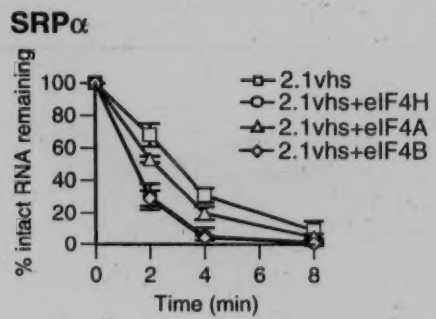

D. SRPα
% intact RNA remaining
100
80
60
40
20
0
0 2 4 6 8
Time (min)
2.1vhs
2.1vhs+eIF4H
2.1vhs+eIF4A
2.1vhs+eIF4B

Figure 3.10: Lower concentrations of eIF4A do not enhance the activity of yeast extracts containing vhs. Quantification of nuclease activity on SRPα RNA. Internally labeled SRPα RNA was added to yeast extracts containing vhs, alone (2.1vhs) or mixed with eIF4A (2.1μM). The quantity of full-length RNA in three experiments was determined using phosphor-imager analysis (Molecular Dynamics) and plotted against time points indicated. The error bars indicate the standard deviation of each time point calculated from at least three independent experiments.

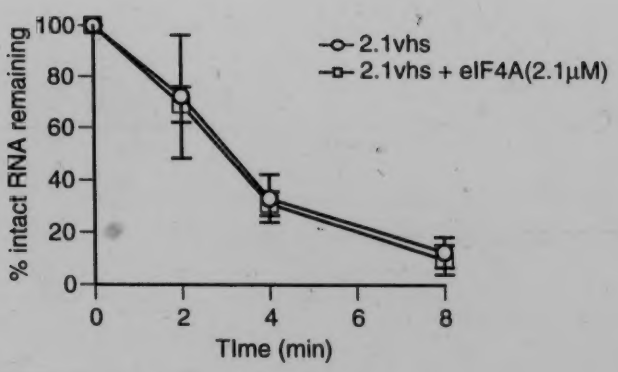

% intact RNA remaining
100
80
60
40
20
0
0
2
4
6
8
TIme (min)
2.1vhs
2.1vhs + eIF4A(2.1μM)

Figure 3.11: Helicase activity of eIF4A. (A) Helicase activity assay. RNA duplexes with a 3' overhang were prepared using RNA_{25}-2 and RNA_{11}-1 oligonucleotides. RNA duplexes were added to reactions which were boiled (boil), lacked ATP (-ATP), lacked eIF4A (-eIF4A) or contained both ATP and eIF4A (+eIF4A). Reactions proceeded for the time indicated and exact details of the assay are described in the Materials and Methods. (B) Quantification of the helicase activity of eIF4A. The ssRNA and dsRNA were quantified using phosphor-imager analysis. Unwinding efficiency was calculated using the following formula : % unwinding = 100 x (counts of ssRNA)/ (counts of ssRNA + counts of duplex). The unwinding efficiency was plotted against the time points indicated.

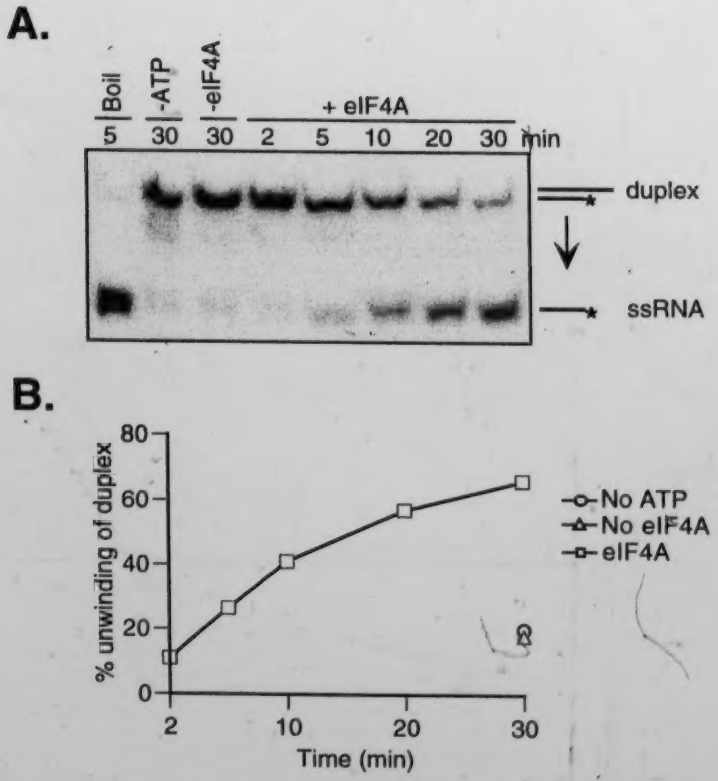
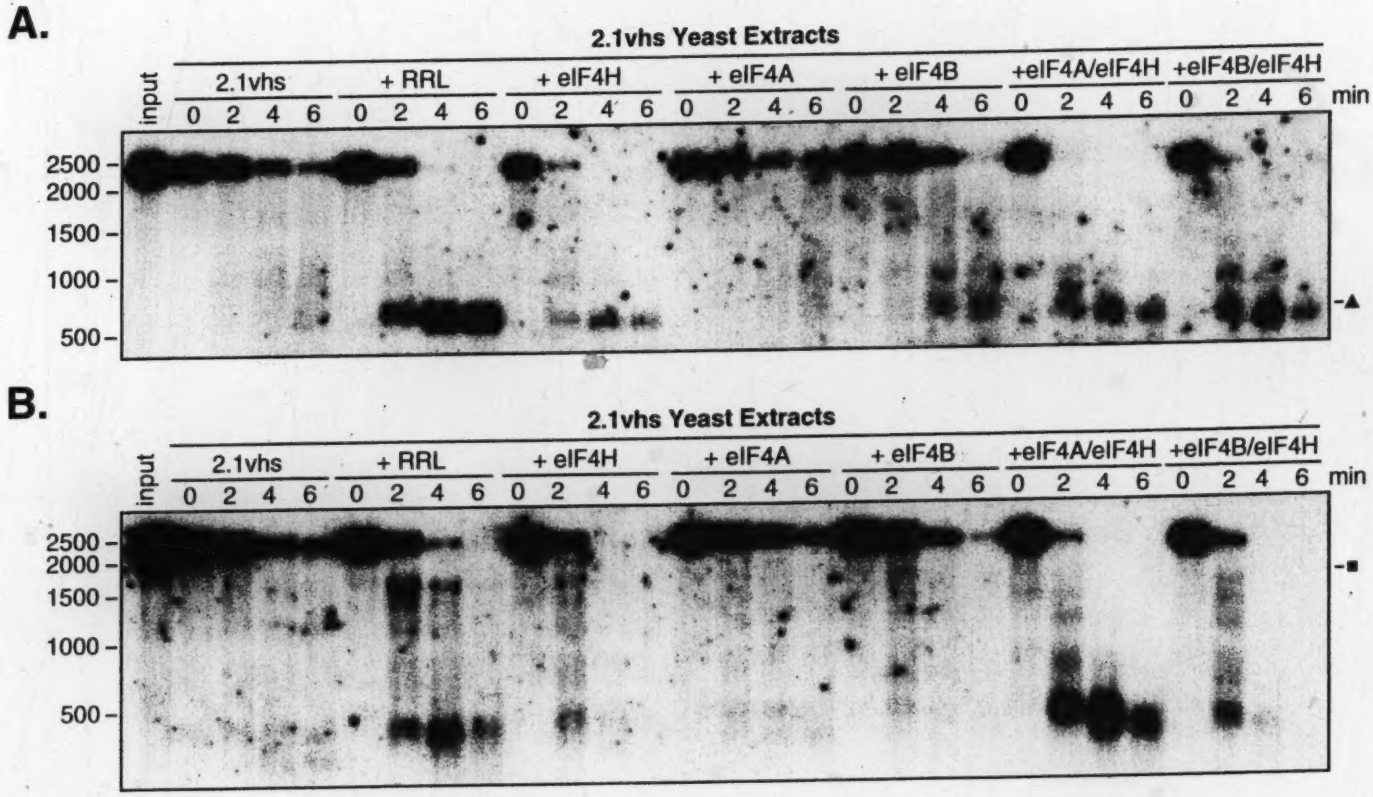

A.
Boil
-ATP
-eIF4A
+ eIF4A
5 30 30 2 5 10 20 30 min
duplex
ssRNA
B.
% unwinding of duplex
80
60
40
20
0
2 10 20 30
Time (min)
No ATP
No eIF4A
eIF4A

Figure 3.12: eIF4A and eIF4B do not restore efficient IRES-directed targeting to yeast expressing vhs. Northern blot analysis of nuclease activity on pCITE-1 RNA using oligonucleotide probes for the 5' (JPP-E, panel A) and 3' (ORD17, panel B) ends of pCITE-1 RNA, respectively. For a diagram of the positions of the oligonucleotides see Figure 3.6, panel B. Unlabeled pCITE-1 RNA was added to yeast extracts containing 2.1vhs, either alone (2.1vhs), or mixed with RRL (+RRL), eIF4H (+eIF4H), eIF4A (+eIF4A), eIF4B (+eIF4B), eIF4A and eIF4H (+eIF4A/eIF4H) and eIF4B and eIF4H (+eIF4B/eIF4H). All proteins was used at a concentration of 2.1 μM. RNA was extracted at the indicated time points, resolved on an agarose-formaldyhyde gel, transferred to a Gene Screen Plus membrane and analyzed by northern blot analysis. The closed triangle indicates the ~ 600-nt IRES-containing fragment, and the closed square indicates the position of the 1800-nt fragment. The position and size of the RNA markers in nucleotides are at the left.


A.
2.1vhs Yeast Extracts
input
2.1vhs
+ RRL
+ eIF4H
+ eIF4A
+ eIF4B
+eIF4A/eIF4H
+eIF4B/eIF4H
0 2 4 6
min
2500
2000
1500
1000
500
B.
2.1vhs Yeast Extracts
input
2.1vhs
+ RRL
+ eIF4H
+ eIF4A
+ eIF4B
+eIF4A/eIF4H
+eIF4B/eIF4H
0 2 4 6
min
2500
2000
1500
1000
500

CHAPTER 4 : DISCUSSION

Previous work, as reviewed in the Introduction, has provided strong evidence that vhs is an integral part of the vhs-dependent RNase, an mRNA-specific nuclease that requires one or more cellular factors for efficient activity. In addition, vhs interacts with translation initiation factor eIF4H (Feng *et al.*, 2001) and a complex of vhs/eIF4H has RNase activity (Everly *et al.*, 2002). However, the role of eIF4H in the activity and selective targeting of the vhs-dependent RNase remained unclear. In this thesis, I demonstrate that cell-free extracts of yeast expressing vhs display vhs-dependent RNase activity in the absence of mammalian factors. This activity differs from that previously described for vhs produced in RRL, in that it was not strongly targeted to sequences located immediately downstream of the EMCV IRES. RRL enhanced the activity of the extracts, and reconstituted IRES-directed targeting to the nuclease. I also found that the eIF4H paralogue, eIF4B, binds to vhs, and eIF4B and eIF4H each stimulate the activity of vhs produced in yeast. However, neither factor was able to fully restore targeting to the EMCV IRES, indicating that one or more other mammalian factors are also required.

The data presented in this thesis clearly demonstrate that cell-free extracts prepared from yeast expressing vhs contain a novel RNase activity that can be detected in the absence of added mammalian factors. This activity has been detected in repeated experiments and with several independent preparations of yeast extracts. However, a previous study from this laboratory did not detect appreciable vhs-dependent RNase activity in extracts of yeast expressing vhs until RRL was added to the extracts (Lu *et al.*, 2001a). The basis for this discrepancy remains unclear. My data suggest that it is not due to drastic differences in the amount of vhs protein in the yeast extracts. Perhaps the RNase activity in extracts is near the lower detection limit of the assay, and our ability to detect it has improved over time. It may also be that currently unknown variations in growth conditions affect the level of activity in the

extracts. Possibly relevant in this context, Lu, *et al.* found that the severity of the growth inhibitory effect of vhs in yeast varies depending on the carbon source used in the medium (Lu *et al.*, 2001a). It is unknown at this time whether vhs expressed in yeast has activity on its own or instead requires interactions with one or more yeast factors. Indeed, *S. cerevisiae* contains a homologue of eIF4H and eIF4B known as Tif3 or Stm1 (Altmann *et al.*, 1993, Coppolecchia *et al.*, 1993). Tif3/Stm1 plays an important role in translation initiation and was found to have 25 % identity and 53 % similarity to the sequence of eIF4H (Richter-Cook *et al.*, 1998). Thus, it is possible that Tif3/Stm1 or other yeast proteins can interact with vhs and form a partially competent RNase.

To date, eIF4H is the only published translation initiation factor to interact with vhs. Therefore, it may be informative to investigate the activity of 2.1vhs extracts derived from a strain of *S. cerevisiae* which contains a disruption in the TIF3/STM1 gene (Altmann *et al.*, 1993, Coppolecchia *et al.*, 1993). If vhs requires Tif3/Stm1 for catalytic activity, one would predict that extracts of ΔTIF3/STM1 yeast expressing vhs would lack detectable RNase activity, which could be restored with the addition of eIF4H or eIF4B. This observation would highlight the necessity of eIF4H/eIF4B for the catalytic activity of the vhs-dependent RNase. Alternatively, if RNase activity is still observed in the yeast extracts, this would suggest that Tif3/Stm1 is not contributing to the RNase activity of vhs, but eIF4H/eIF4B should still enhance the activity of 2.1vhs. The tandem affinity purification method is a procedure to purify protein complexes from yeast under native conditions that can then be analyzed by mass spectrometry for interacting components (Puig *et al.*, 2001). This procedure could be used to determine the subunit composition of the vhs-dependent RNase present in yeast extracts and thus, potential mammalian factors that interact with vhs.

The vhs-dependent nuclease activity observed in yeast extracts may explain the previous finding that vhs strongly inhibits the growth of yeast on certain carbon sources, an effect that correlates with the mammalian host

shutoff activity of vhs in mutational studies (Lu *et al.*, 2001a). However, this hypothesis is not obviously consistent with the observation that vhs does not induce global turnover of yeast mRNAs *in vivo* (Lu *et al.*, 2001a). Perhaps the RNase acts only on a subset of yeast mRNAs *in vivo*, or the effect is too weak to be detected by northern blot analysis. An interesting alternative possibility is that vhs inhibits the growth of yeast by binding the eIF4H homologue Tif3/Stm1 and blocking its activity, rather than by (or in addition to) destabilizing mRNAs. Indeed TIF3/STM1 gene disruptions cause a slow growth phenotype and a defect in translation initiation (Altmann *et al.*, 1993, Coppolecchia *et al.*, 1993). Further studies are required to determine if the inhibition of Tif3/Stm1 contributes to the growth inhibitory effect of vhs in yeast. The effect of vhs on the growth rate of a *tif3/stm1* mutant yeast strain (Altmann *et al.*, 1993, Coppolecchia *et al.*, 1993) could be tested. If the expression of vhs does not have an effect on the growth rate of the *tif3/stm1* mutant, then the simplest explanation is that vhs is only interacting with Tif3/Stm1 and inhibiting its function. This could be confirmed by transfecting in the TIF3/STM1 gene and demonstrating no change in the growth rate. However, if the expression of vhs further attenuates the growth rate of the *tif3/stm1* mutant and does not affect the stability of yeast mRNAs, then vhs may be interacting with other yeast factors. If the subunit composition of the vhs-dependent RNase has been determined, other disruption mutant yeast strains could be tested, with both individual and combination disruptions.

The RNase activity detected in yeast extracts differed in several respects from that of vhs translated in RRL. Previous work has shown that the activity in RRL is non-randomly targeted relative to the structure of the substrate mRNA. Thus, the initial sites of cleavage on SRPα mRNA are clustered over the 5' quadrant of the transcript (Elgadi *et al.*, 1999), and the EMCV IRES present in pCITE-1 RNA targets the initial cleavage events to 3' flanking sequences (Elgadi & Smiley, 1999). In addition, as noted above, our unpublished data strongly suggest that the subsequent degradation of pCITE-1 RNA (mediated by

vhs, (Lu *et al.*, 2001b)) proceeds in an overall 5' → 3' direction in the RRL system (Perez-Prada and Smiley, unpublished). In this thesis, the detailed mode of decay of SRPα mRNA in the yeast extract system was not examined, and therefore it is not known whether the nuclease activity is initially targeted to the 5' end of this RNA. However, determining if vhs produced in yeast has the ability to target to the 5' end of SRPα RNA is important, as it is entirely possible that 2.1vhs still retains this ability or that eIF4H/eIF4B can affect cap-dependent targeting.

In contast, my data provide a clear indication that the 2.1vhs-dependent RNase is largely untargeted relative to the EMCV IRES, with no obvious strong clustering of preferred initial cleavage sites immediately downstream of the IRES in the pCITE-1 transcript. In addition, while discrete degradation products were produced from pCITE-1 RNA at relatively low molar yield, they did not appear in the defined temporal order indicative of initial IRES targeting and subsequent 5' → 3' decay as observed in RRL. Moreover, the 600-nt IRES-containing fragment was relatively unstable in the yeast extracts. It is unclear why the molar yield of the discrete degradation intermediates of pCITE-1 RNA was reduced in the yeast extracts relative to reactions supplemented with RRL, eIF4H, or eIF4B. It is possible that the vhs-dependent nuclease cleaves the substrate at many more different sites in the absence of mammalian factors, or acts primarily as an exonuclease. Alternatively, the degradation intermediates may be more susceptible to yeast nucleases if the IRES is normally stabilized by a factor(s) in RRL that is not present in the yeast extracts. The influence of RRL, eIF4B or eIF4H on the overall polarity of RNA degradation by 2.1vhs was not specifically addressed in this thesis. However, there is some indication that the addition of RRL to the yeast extracts enhanced the 5' → 3' decay of pCITE-1 RNA. The use of oligonucleotides complementary to sequences along the length of pCITE-1 and SRPα RNAs will yield a clearer picture of the tracking ability of the vhs-dependent RNase, in the presence or absence of mammalian factors.

It is interesting to note that the inherent preference of the vhs-dependent nuclease for RNA substrates is different in the yeast and RRL systems. In RRL, the vhs-dependent RNase has a much stronger preference for IRES-bearing RNA substrates (Lu *et al.*, 2001b). pCITE-1 RNA is degraded significantly faster than SRPα RNA in RRL when lower concentrations of vhs protein are used (Lu *et al.*, 2001b). However in the yeast system, this preference appears to be reversed. In 2.1vhs yeast extracts, SRPα RNA is degraded faster than pCITE-1 RNA, though the difference between the rates of degradation is variable between batches of yeast extracts. The significant amount of secondary structure in the IRES element at the 5' end of pCITE-1 RNA may block the vhs-dependent RNase from loading onto the substrate. Thus, SRPα RNA, which does not contain an IRES at its 5' end, may now be more susceptible to vhs-dependent cleavage in yeast. The use of additional RNA substrates may clarify the discrepancy between the RRL and yeast systems. Two RNA substrates which could be used are: pCITE-1 Msc/RI, in which the EMCV IRES was deleted from pCITE-1 RNA (Elgadi & Smiley, 1999); and an RNA substrate in which the EMCV IRES was inserted into the very 5' end of SRPα RNA. These RNAs would help decipher if SRPα RNA is degraded faster in yeast due to the lack of a 5' IRES.

The data presented in this thesis demonstrate that mammalian factors significantly alter the characteristics of the nuclease activity of vhs expressed in yeast. Thus, RRL restored IRES-directed targeting and (as previously reported, (Lu *et al.*, 2001a)) enhanced the RNase activity of yeast extracts containing vhs. In contrast, eIF4B and eIF4H stimulated the activity of vhs without fully restoring IRES-mediated targeting. The pattern of RNA cleavage products when eIF4H or eIF4B was added to yeast extracts containing vhs was very different from that seen when RRL was used. On the pCITE-1 RNA substrate, eIF4H and eIF4B cleave both immediately 3' downstream of the IRES and at sites along the length of the RNA. The nature and position of these novel cleavage sites could

be further investigated using primer extension analysis of the major cleavage products.

There was initially some concern over the observed nuclease activity in the protein preparations of the partially purified translation initiation factors. However, a number of control experiments were performed to confirm that the stimulation of nuclease activity of 2.1vhs is due to eIF4H or eIF4B and not a contaminating bacterial nuclease. I found that eIF4H and eIF4B did not induce nuclease activity when added to empty vector yeast extracts, the contaminating nuclease activity could be quenched with the addition of RNase-free BSA and total yeast mRNA, and a non-relevant protein cdc34Δ_{209} did not enhance the nuclease activity of the 2.1vhs yeast extracts. Additionally, Wei-Li Hsu, a post doc in our laboratory has made preparations of eIF4H which do not contain contaminating nuclease activity and Holly Saffron, a technician in our laboratory, has found that these preparations function identically to the eIF4H preparations used in the experiments for this thesis. Thus, nuclease-free eIF4H stimulates the RNase activity of vhs on both pCITE-1 and SRPα RNA.

While it appears clear that eIF4H and eIF4B are stimulating the nuclease activity of the 2.1vhs extracts, it is possible that the nuclease activity observed is not due to 2.1vhs but rather a yeast nuclease. Vhs expression in yeast causes an inhibition of growth (Lu *et al.*, 2001a) and therefore, the possibility exists that the expression of yeast proteins is altered in vhs expressing yeast. Consequently, the 2.1vhs extracts may contain yeast nucleases, that can be stimulated by RRL, eIF4H or eIF4B, at a much higher levels than in yeast which contain the empty vector. A control that could be used is the vhs mutant allele D215N, which contains a point mutation in a potential catalytic residue (Everly *et al.*, 2002), but still retains the ability to interact with eIF4H (Feng *et al.*, 2001) (Everly *et al.*, 2002). D215N cannot inhibit the expression of a cotransfected reporter gene in Vero cells (Everly *et al.*, 2002, Feng *et al.*, 2001) and can be purified as a nuclease-deficient complex of D215N/eIF4H from *E. coli* (Everly *et al.*, 2002). The expression of D215N in yeast may mimic wild-type vhs

expression more closely, as D215N can interact with eIF4H, and therefore secondary effects that are not due to the nuclease activity of 2.1vhs may be similar for 2.1vhs and D125N. Holly Safron, from our lab has assayed extracts of yeast expressing D215N and found they had very little nuclease activity which was unable to be enhanced by the addition of RRL or eIF4H.

Feng, *et al.* demonstrated that the vhs-binding site on eIF4H lies within amino acids 90-137, however the eIF4H binding site on vhs was not conclusively determined and appears to involve at least two domains of the protein (Feng *et al.*, 2001). The vhs interaction site on eIF4B must still be defined and determined if it is in the region of homology between eIF4H and eIF4B. Mutagenesis and systematic deletions of the vhs protein may more precisely define the domains required for interaction with eIF4H and eIF4B. It will be interesting to determine if any differences can be found between eIF4H and eIF4B in their interaction with vhs. Once the minimum vhs-binding fragment of eIF4B has been determined, both eIF4H and eIF4B can be tested to see if the minimum vhs-binding fragment is sufficient to stimulate the activity of vhs expressed in yeast. If not, progressively larger fragments could be tested. When the minimum vhs stimulating domain of eIF4H and eIF4B is found, these can be used in competition binding assays to determine if either eIF4H or eIF4B can out compete the other for binding, thus determining if either one or the other binds vhs with a higher affinity.

Compared to eIF4HeIF4B, the role, if any, of eIF4A in the vhs-dependent RNase is much less clear. eIF4A does not interact with vhs using far western analysis, and could not restore IRES-targeting to yeast extracts containing vhs. However, when eIF4A was added to 2.1vhs extracts at a high concentration (7.6 μM), an enhancement of nuclease activity was observed on SRPα RNA but not pCITE-1 RNA. When the concentration of eIF4A was lowered (2.1 μM), the effect on SRPα RNA subsequently disappeared, while at the same concentration, eIF4B and eIF4H strongly enhanced the RNase activity of 2.1vhs. In addition, while I did not detect an interaction, recently using the yeast

two-hybrid system and GST pull-downs vhs was found to interact with eIF4A (D.N. Everly, 26th International Herpesvirus Workshop). The interaction domain appears to differ from that which interacts with eIF4H, as some vhs mutants which do not interact with eIF4H still interact with eIF4A (D.N. Everly, 26th International Herpesvirus Workshop). It is plausible that in the yeast extracts, human eIF4A binds vhs, interacts with the yeast translational machinery, and thus delivers vhs to areas of cap-dependent translation initiation. Effectively, eIF4A may be enhancing the nuclease activity of 2.1vhs by increasing the targeting of 2.1vhs to the 5' end of SRPα RNA. It has recently been found that the yeast eIF4A and eIF4G proteins interact (Dominguez *et al.*, 1999, Neff & Sachs, 1999), and mammalian eIF4A can bind yeast eIF4G1 (Dominguez *et al.*, 2001). However, mammalian eIF4A cannot substitute for yeast eIF4A both *in vivo* and in the yeast *in vitro* translation system (Dominguez *et al.*, 2001, Prat *et al.*, 1990). However, while this may discredit a role for eIF4A, it is important to note that vhs does not require ribosomes for nuclease activity *in vitro* (Elgadi *et al.*, 1999, Elgadi & Smiley, 1999, Sorenson *et al.*, 1991), and vhs-dependent nuclease activity still persists in the presence of translation inhibitors (Fenwick & McMenamin, 1984, Schek & Bachenheimer, 1985, Strom & Frenkel, 1987). Thus, eIF4A can potentially enhance the nuclease activity of vhs on SRPα RNA without the need for translation to occur, simply by the delivery of 2.1vhs by human eIF4A, through an interaction with yeast eIF4G, to the 5' end of SRPα RNA. To determine if an increase in 5' targeting occurs when human eIF4A is added to the 2.1vhs extracts, one could monitor the 5' and 3' ends of SRPα RNA during the reaction and use primer extension analysis on the smaller degradation fragments to determine where the initial cleavages occur.

It is perhaps not surprising that vhs requires mammalian factors to target immediately downstream of the EMCV IRES, as this IRES cannot direct translation initiation in yeast (Evstafieva *et al.*, 1993). Although the basis for this inactivity is unclear, a likely possibility is that yeast translation initiation factors cannot functionally interact with the IRES or the IRES requires mammalian

factors to maintain its secondary structure for recruitment of translation factors. RRL fully reconstituted IRES-directed targeting to vhs expressed in yeast, while eIF4B and eIF4H did not, suggesting that distinct, or multiple, mammalian factors are required for the targeting reaction. The combination of eIF4A and eIF4H was also inactive, and a role for eIF4H in IRES-directed targeting is perhaps unlikely given that a mutant form of vhs (vhs1, T214→I (Kwong *et al.*, 1988, Read & N., 1983)) that no longer interacts with eIF4H (Feng *et al.*, 2001), retains the ability to perform IRES-directed cleavage (Lu *et al.*, 2001b). However, the combination of eIF4A and eIF4B was not tested. It will be important to do so in future studies, as eIF4A and eIF4B together greatly enhance the binding of each other to the EMCV IRES (Pestova *et al.*, 1996b). An additional approach to the vhs activity assay in yeast would be to immunodeplete RRL using antibodies against eIF4A and eIF4B which have been described in the literature (Pestova *et al.*, 1996b). The immunodelpeted RRL could then be used to test *in vitro* translated vhs for IRES-targeting, and for use in the yeast vhs activity assay. If this approach yields a negative result, one could test chromatographic fractions of RRL for the ability to restore nuclease activity to vhs expressed in yeast in order to isolate the proteins needed by the vhs-dependent RNase.

eIF4G also plays a strong role in recruiting translation factors and promoting ribosomal attachment to the IRES (see references in (Kolupaeva *et al.*, 2003)). The binding of eIF4A, eIF4B and eIF4G to the EMCV IRES is strongly stimulated by the presence of all three factors (Lomakin *et al.*, 2000, Pestova *et al.*, 1996b), and the IRES has evolved to bind the eIF4G/eIF4A complex with high affinity rather than eIF4G alone (Lomakin *et al.*, 2000). Thus, it is reasonable to suggest that a combination of all three factors may be required for IRES-directed targeting by vhs. However, the role of eIF4A and eIF4G may be more important than eIF4B as the *vhs*1 mutant, which does not bind to eIF4B, but still interacts with eIF4A (G.S. Read, personal communication), can still mediate efficient IRES-targeting (Lu *et al.*, 2001b). To

test this possibility, purified eIF4G could be obtained and combinations of all three factors in the yeast vhs activity assay could be tested. Holly Saffron, a technician in our laboratory has found that a mutant EMCV IRES, GW8 (Witherell *et al.*, 1995), which destroys most of the J-K domain and no longer binds eIF4G (Kolupaeva *et al.*, 1998, Pestova *et al.*, 1996a), does not promote IRES-targeting by vhs translated in RRL. However, a smaller mutation in the EMCV IRES, 768Δ4 (Hoffman & Palmenberg, 1995), which deletes four bases in the A-bulge and disrupts binding of eIF4G (Pestova *et al.*, 1996a), still induces targeting by RRLvhs. Thus, additional factors may also be required, such as other canonical translation initiation factors and/or IRES trans-acting factors (ITAFs). However, 768Δ4 may still retain some residual eIF4G-binding which was not detected by primer extension analysis. An important experiment will be to determine exactly what initiation factors can interact with each mutant IRES.

Another factor which may be involved is the pyrimidine-tract binding protein (PTB) which helps maintain higher-order structure in the EMCV IRES under non-ideal conformation situations (Kaminski & Jackson, 1998) and is thought to be required for efficient IRES-driven translation *in vivo* (Kaminski & Jackson, 1998) (Kim & Jang, 1999). PTB is an ITAF that binds to six sites on the EMCV IRES (Kolupaeva *et al.*, 1996), two of which are situated in the K domain, one of the domains disrupted in GW8, the IRES that did not induce targeting by vhs. PTB can be easily depleted from RRL using commercially available poly (U)-Sepharose. The PTB depleted RRL and purified recombinant PTB could then be used to study vhs IRES-targeting. Other factors which may be involved in IRES-targeting by vhs are two ITAFs that bind the EMCV IRES but are not required for translation *in vitro*, the La protein (Kim & Jang, 1999), and ITAF45 (Hellen & Sarnow, 2001). One potentially relevant canonical initiation factor is eIF3, as it binds to both eIF4B (Methot *et al.*, 1996b), and eIF4G (Lamphear *et al.*, 1995) and there is some evidence to suggest that it may be able to interact with the IRES directly without the need for eIF4G

(Lomakin *et al.*, 2000). Further studies using purified proteins are required to determine the precise interplay of factors required for IRES-directed targeting by vhs. The vhs-dependent RNase could be purified from both RRL and mammalian cells using the tandem affinity purification method and mass spectrometry to determine what factors interact with the vhs protein.

The data presented in this thesis demonstrate that eIF4B and eIF4H are each able to stimulate the nuclease activity of vhs expressed in yeast. However, the mechanism of this enhancement remains to be defined. It seems likely that the stimulatory effect is a consequence of the direct interactions between the translation factors and vhs (however see below). In addition, it seems plausible that eIF4B and eIF4H stimulate vhs activity through similar mechanisms, given the functional and sequence similarities between these proteins. The normal function of eIF4B and eIF4H in the cell revolves around the RNA helicase eIF4A (Rogers *et al.*, 2002). In the absence of eIF4H and eIF4B, eIF4A (or eIF4F) acts as a non-processive ATP-dependent helicase (Rogers *et al.*, 1999). eIF4B stimulates the helicase activity of eIF4A, at least in part by increasing the affinity of eIF4A for RNA and by increasing the utilization of ATP by eIF4A (Abramson *et al.*, 1987, Abramson *et al.*, 1988, Bi *et al.*, 2000). eIF4H also stimulates the helicase activity of eIF4A (Richter *et al.*, 1999, Rogers *et al.*, 1999), but only slightly enhances the affinity of eIF4A for RNA and does not enhance the catalytic step of ATP hydrolysis by eIF4A (Richter *et al.*, 1999, Richter-Cook *et al.*, 1998). eIF4H has been proposed to stabilize conformational changes that eIF4A cycles through during the helicase reaction (Richter *et al.*, 1999). Furthermore, eIF4A (or eIF4F) becomes a slightly processive helicase in the presence of eIF4B or eIF4H (Rogers *et al.*, 1999) (Rogers *et al.*, 2001), and these two proteins have an additive, rather than synergystic effect on eIF4A when both are included in the helicase reaction (Rogers *et al.*, 1999, Rogers *et al.*, 2001).

In light of this information there are at least three distinct ways that the physical interaction between vhs and eIF4B/eIF4H could enhance the nuclease

activity of vhs. First, by directly binding to vhs, eIF4H and eIF4B may induce a conformational change in either vhs or the complex to create a more active nuclease. The active site, contained at least in part by vhs (Everly *et al.*, 2002) may not be in a favorable conformation or may be partially hidden and require the binding of eIF4H or eIF4B to assume an optimal configuration. Interestingly, the activity of a homolgue of vhs, 5' flap endonuclease/5'-3' exonuclease (FEN-1), is affected by an interaction with two proteins. The Werner Syndrome protein (WRN), interacts with FEN-1 (Brosh *et al.*, 2001) and stimulates the FEN-1 cleavage reaction by increasing the reaction velocity (Brosh *et al.*, 2002). The proliferating cell nuclear antigen (PCNA) protein also binds to FEN-1 and acts to stabilize FEN-1 binding to the substrate (Tom *et al.*, 2000). Second, eIF4B and eIF4H may enhance the affinity of vhs or the complex for the RNA substrate, either by inducing a conformational change in vhs or by directly contributing to RNA binding. Indeed, eIF4B and eIF4H each contain a conserved RNA recognition motif (RRM, see for example (Methot *et al.*, 1994, Richter-Cook *et al.*, 1998)) that might direct the complex to particular RNA sequences and/or enhance its general affinity for RNA. Third, the factors might act to alter the overall pattern of RNase activity, for example by triggering a shift from a distributive to a processive mode of RNA degradation (or *vice-versa*). Enzyme kinetic studies will help determine the function of eIF4H and eIF4B in the vhs cleavage reaction.

In this latter context, it is interesting to note that vhs is required both for the initial cleavage of mRNAs and the subsequent degradation of the decay products in the RRL assay system (Lu *et al.*, 2001b). Moreover, as noted above, the subsequent decay of the initial 3' cleavage product of pCITE-1 mRNA appears to proceed 5' → 3' (Perez-Prada and Smiley, unpublished). Both observations are consistent with a model in which vhs is a processive enzyme that, once loaded, tracks along the RNA, cleaves it at particular sites, and remains associated with the 3' cleavage product. Alternatively, vhs may be a distributive enzyme with a preference for the 5' ends of the mRNA degradation

intermediates. Some indirect evidence is consistent with a role of eIF4H (and by analogy, perhaps eIF4B) in the decay of the first products of vhs action. Specifically, as noted above, the vhs1 mutant version of vhs (T214→I), which is defective in host shutoff *in vivo* (Kwong *et al.*, 1988, Read & N., 1983) and unable to degrade SRPα RNA *in vitro* (Elgadi *et al.*, 1999), does not detectably interact with eIF4H (Feng *et al.*, 2001). The vhs1 protein nevertheless retains the ability to perform IRES-directed cleavage of pCITE-1 RNA in the RRL system (Lu *et al.*, 2001b). However, the resulting 3' 1800-nt fragment is not subject to further decay, suggesting that the mutation severely impairs the ability of vhs to continue degradation of the RNA after the first cleavage reaction (Lu *et al.*, 2001b). Thus, considered in the light of the foregoing, eIF4H may enhance the decay of the initial degradation products by increasing the processivity of vhs or by promoting its distributive cycling on and off the substrate. Further analysis of the vhs nuclease reaction using purified vhs will be required to denote between the possibilities.

The three scenarios described above all assume that eIF4B and eIF4H stimulate vhs activity in yeast extracts solely through their direct interactions with vhs. However, it is important to stress that this is not necessarily the case. As mentioned above, mammalian eIF4A can interact with yeast eIF4G (Dominguez *et al.*, 2001), and mammalian eIF4B can functionally replace yeast Tif3/Stm1 in *in vitro* translation reactions (Altmann *et al.*, 1993) and are thus capable of interacting with other components of the yeast translational apparatus. The same may also be so for eIF4H. Therefore, it possible that some or all of the effects of eIF4B and eIF4H that I have observed, stem from the recruitment of additional yeast proteins into the vhs nuclease complex. As mentioned above, purifying the vhs-dependent RNase from yeast will determine what yeast factors are involved and could be tested for their effects on the vhs-dependent RNase.

The study of vhs has been hampered by the inability to purify the protein. The protein when expressed in *E. coli* is very insoluble and attempts to

solubilize the protein have resulted in a non-functional protein preparation (Everly *et al.*, 2002). Everly *et al.* was able to co-express eIF4H and vhs together in *E. coli* and partially purify the complex (Everly *et al.*, 2002). However, the complex was not produced in great quantity and the protein preparations were still contaminated with a number of bacterial proteins. The purification of vhs in complex with eIF4A may be a better alternative. eIF4A has been found to interact with vhs (D.N. Everly, 26th International Herpesvirus Workshop), and I found that eIF4A does not substantially enhance the nuclease activity of vhs. Therefore, the co-expression of vhs and eIF4A in *E. coli*, should allow the purification of a soluble vhs/eIF4A complex which may be expressed at a much higher level than vhs/eIF4H due to a lower or lack of nuclease activity. In addition, the use of this complex would eliminate the complicating issue of yeast factors, and the use of purified translation factors would allow a rapid determination of factors required for wild-type vhs activity.

All of the experiments presented in this thesis were performed *in vitro*, thus the *in vivo* relevance of eIF4H and eIF4B for vhs function remains unclear. There is some evidence for the biological relevance of the vhs–eIF4H/eIF4B interaction. A number of mutants were found which lacked vhs activity in transient co-transfection assays and were unable to interact with eIF4H (Feng *et al.*, 2001). Thus, there appears to be a correlation between the inability to interact with eIF4H and a lack of vhs activity. One of these mutants, is the *vhs*1 mutant (Read & N., 1983), which is a spontaneous mutant, and is unable to mediate the early shutoff of host protein synthesis in tissue culture (Read & N., 1983). In addition, the *vhs*1 mutant shows a 5- to 10-fold reduction in titer (Read & N., 1983), and displays little if any mRNA degradation of SRPα RNA (Elgadi *et al.*, 1999). To further investigate the *in vivo* relevance of eIF4B and eIF4H, co-transfection assays could be performed in tissue culture cells in which the expression of the translation factors is blocked by RNAi. Interestingly, when the tissue distribution of eIF4H and eIF4B mRNA was analyzed, it was found that both mRNAs were present in most of the tissues investigated (Osborne *et al.*,

1996, Richter *et al.*, 1999). However, the mRNA levels differed relative to one another. So, for example, in the brain, eIF4H mRNA was expressed 2-fold higher than eIF4B, and in skeletal muscle, eIF4B mRNA was expressed 3.7-fold higher than eIF4H (Richter *et al.*, 1999). These observations suggest that there may be tissue-specific functions of the two proteins. Determining the mRNA and protein levels of eIF4B and eIF4H in mucosal epithelial tissue may provide insight as to the relative importance of the factors to the vhs-dependent RNase.

Future studies in vhs research are headed in three main directions. The first main focus is the further investigation of the biological role of vhs. Recent studies have suggested a role for vhs in the evasion of host immune responses. Vhs has been implicated in the loss of MHC class I (Tigges *et al.*, 1996), and class II molecules (Trgovcich *et al.*, 2002) from the cell surface, in IFN resistance (Murphy *et al.*, 2003) and in the inactivation of dendritic cells (Samady *et al.*, 2003). Some of the future issues to be addressed will be to identify if vhs can inhibit antiviral responses in the animal model and which pathways are affected, and determine if the evasion of immune responses is solely due to the RNase activity of vhs. A second focus is investigating the *in vivo* mechanism of vhs action. This includes, determining the requirements of vhs for mRNA recognition (for example, are a cap or poly(A) tail required), and the direction of the cleavage reaction (5'→3' or 3'→5'). A third main focus of vhs research will be to further investigate the role of translation initiation factors (including eIF4A, eIF4B, and eIF4H) in the activity and targeting of vhs.

The data presented in this thesis demonstrate that mammalian factors alter both the RNase activity and IRES-targeting properties of vhs. The *in vitro* assay described here will facilitate future studies designed to identify the factors required for IRES-directed targeting and illuminate the mechanisms that regulate vhs activity.

CHAPTER 5 : REFERENCES

Abramson, R. D., Dever, T. E., Lawson, T. G., Ray, B. K., Thach, R. E. & Merrick, W. C. (1987). The ATP-dependent interaction of eukaryotic initiation factors with mRNA. *J Biol Chem* 262, 3826-32.

Abramson, R. D., Dever, T. E. & Merrick, W. C. (1988). Biochemical evidence supporting a mechanism for cap-independent and internal initiation of eukaryotic mRNA. *J Biol Chem* 263, 6016-9.

Ace, C. I., Dalrymple, M. A., Ramsay, F. H., Preston, V. G. & Preston, C. M. (1988). Mutational analysis of the herpes simplex virus type 1 trans-inducing factor Vmw65. *Journal of General Virology* 69, 2595-2605.

Advani, S. J., Brandimarti, R., Weichselbaum, R. R. & Roizman, B. (2000a). The disappearance of cyclins A and B and the increase in activity of the G(2)/M-phase cellular kinase cdc2 in herpes simplex virus 1-infected cells require expression of the alpha22/U(S)1.5 and U(L)13 viral genes. *J Virol* 74, 8-15.

Advani, S. J., Weichselbaum, R. R. & Roizman, B. (2000b). The role of cdc2 in the expression of herpes simplex virus genes. *Proc Natl Acad Sci U S A* 97, 10996-1001.

Advani, S. J., Weichselbaum, R. R. & Roizman, B. (2001). cdc2 cyclin-dependent kinase binds and phosphorylates herpes simplex virus 1 U(L)42 DNA synthesis processivity factor. *J Virol* 75, 10326-33.

Advani, S. J., Weichselbaum, R. R. & Roizman, B. (2003). Herpes simplex virus 1 activates cdc2 to recruit topoisomerase II alpha for post-DNA synthesis expression of late genes. *Proc Natl Acad Sci U S A* 100, 4825-30.

Altmann, M., Muller, P. P., Wittmer, B., Ruchti, F., Lanker, S. & Trachsel, H. (1993). A Saccharomyces cerevisiae homologue of mammalian translation initiation factor 4B contributes to RNA helicase activity. *EMBO J.* 12, 3997-4003.

Alwine, J. C., Steinhart, W. L. & Hill, C. W. (1974). Transcription of herpes simplex type 1 DNA in nuclei isolated from infected HEp-2 and KB cells. *Virology* 60, 302-307.

Anderson, K. P., Frink, R. J., Devi, G. B., Gaylord, B. H., Costa, R. H. & Wagner, E. K. (1981). Detailed characterization of the mRNA mapping in the HindIII fragment K region of the herpes simplex virus type 1 genome. *J Virol* 37, 1011-27.

Aubert, M. & Blaho, J. A. (1999). The herpes simplex virus type 1 regulatory protein ICP27 is required for the prevention of apoptosis in infected human cells. *J Virol* 73, 2803-13.

Bailey, D. & O'Hare, P. (2002). Herpes simplex virus 1 ICP0 co-localizes with a SUMO-specific protease. *J. Gen. Virol.* 83, 2951-64.

Baines, J. D. & Roizman, B. (1992). The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. *J Virol* 66, 5168-74.

Banfield, B. W., LeDuc, Y., Esford, L., Schubert, K. & Tufaro, F. (1995). Sequential isolation of proteoglycan synthesis mutants by using herpes simplex virus as a selective agent: evidence for a proteoglycan-independent virus entry pathway. *J. Virol.* 69, 3290-3298.

Batterson, W., Furlong, D. & Roizman, B. (1983). Molecular genetics of herpes simplex virus. VII. Further characterization of a ts mutant defective in release of viral DNA and in other stages of viral reproductive cycle. *J. Virol.* 45, 397-407.

Batterson, W. & Roizman, B. (1983). Characterization of the herpes simplex virus virion associated factor responsible for the induction of alpha genes. *J. Virol.* 46, 371-377.

Beard, P. M., Taus, N. S. & Baines, J. D. (2002). DNA cleavage and packaging proteins encoded by genes U(L)28, U(L)15, and U(L)33 of herpes simplex virus type 1 form a complex in infected cells. *J Virol* 76, 4785-91.

Becker, Y., Dym, H. & Sarov, I. (1968). Herpes simplex virus DNA. *Virology* 36, 184-192.

Bi, X., Ren, J. & Goss, D. J. (2000). Wheat germ translation initiation factor eIF4B affects eIF4A and eIFiso4F helicase activity by increasing the ATP binding affinity of eIF4A. *Biochemistry* 39, 5758-65.

Blaho, J. A., Zong, C. S. & Mortimer, K. A. (1997). Tyrosine phosphorylation of the herpes simplex virus type 1 regulatory protein ICP22 and a cellular protein which shares antigenic determinants with ICP22. *J Virol* 71, 9828-32.

Blau, J., Xiao, H., McCracken, S., O'Hare, P., Greenblatt, J. & Bentley, D. (1996). Three functional classes of transcriptional activation domains. *Mol. Cell Biol.* 16, 2044-2055.

Boutell, C., Sadis, S. & Everett, R. D. (2002). Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. *J Virol* 76, 841-50.

Brosh, R. M., Jr., Driscoll, H. C., Dianov, G. L. & Sommers, J. A. (2002). Biochemical characterization of the WRN-FEN-1 functional interaction. *Biochemistry* 41, 12204-16.

Brosh, R. M., Jr., von Kobbe, C., Sommers, J. A., Karmakar, P., Opresko, P. L., Piotrowski, J., Dianova, I., Dianov, G. L. & Bohr, V. A. (2001). Werner syndrome protein interacts with human flap endonuclease 1 and stimulates its cleavage activity. *Embo J* 20, 5791-801.

Bruce, J. W. & Wilcox, K. W. (2002). Identification of a motif in the C terminus of herpes simplex virus regulatory protein ICP4 that contributes to activation of transcription. *J Virol* 76, 195-207.

Bryant, H. E., Wadd, S. E., Lamond, A. I., Silverstein, S. J. & Clements, J. B. (2001). Herpes simplex virus IE63 (ICP27) protein interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step. *J Virol* 75, 4376-85.

Cai, W. & Schaffer, P. (1992). Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. *J. Virol.* 66, 2904-2915.

Campbell, M. E. M., Palfreyman, J. W. & Preston, C. M. (1984). Identification of herpes simplex virus DNA sequences which encode a *trans*-acting polypeptide responsible for stimulation of the immediate early transcription. *J. Mol. Biol.* 180, 1-9.

Carrozza, M. J. & DeLuca, N. (1998). The high mobility group protein 1 is a coactivator of herpes simplex virus ICP4 in vitro. *J Virol* 72, 6752-7.

Carrozza, M. J. & DeLuca, N. A. (1996). Interaction of the viral activator protein ICP4 with TFIID through TAF250. *Mol. Cell. Biol.* 16, 3085-3093.

Carter, K. L. & Roizman, B. (1996). Alternatively spliced mRNAs predicted to yield frame-shift proteins and stable intron 1 RNAs of the herpes simplex virus 1 regulatory gene alpha 0 accumulate in the cytoplasm of infected cells. *Proc. Natl. Acad. Sci. USA* 93, 12535-12540.

Challberg, M. D. (1986). A method for identifying the viral genes required for herpesvirus DNA replication. *Proc. Natl. Acad. Sci., USA* 83, 9094-9098.

Chee, A. V., Lopez, P., Pandolfi, P. P. & Roizman, B. (2003). Promyelocytic leukemia protein mediates interferon-based anti-herpes simplex virus 1 effects. *J Virol* 77, 7101-5.

Chelbi-Alix, M. K. & de The, H. (1999). Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. *Oncogene* 18, 935-41.

Chen, I. H., Sciabica, K. S. & Sandri-Goldin, R. M. (2002). ICP27 interacts with the RNA export factor Aly/REF to direct herpes simplex virus type 1 intronless mRNAs to the TAP export pathway. *J Virol* 76, 12877-89.

Chen, Y. M. & Knipe, D. M. (1996). A dominant mutant form of the herpes simplex virus ICP8 protein decreases viral late gene transcription. *Virology* 221, 281-90.

Cheung, P., Ellison, K. S., Verity, R. & Smiley, J. R. (2000). Herpes simplex virus ICP27 induces cytoplasmic accumulation of unspliced polyadenylated alpha-globin pre-mRNA in infected HeLa cells. *J Virol* 74, 2913-9.

Cocchi, F., Menotti, L., Mirandola, P., Lopez, M. & Campadelli-Fiume, G. (1998). The ectodomain of a novel member of the immunoglobulin subfamily related to the poliovirus receptor has the attributes of a bona fide receptor for herpes simplex virus types 1 and 2 in human cells. *J Virol* 72, 9992-10002.

Cook, W. J., Lin, S. M., DeLuca, N. A. & Coen, D. M. (1995). Initiator elements and regulated expression of the herpes simplex virus thymidine kinase gene. *J Virol* 69, 7291-4.

Coppolecchia, R., Buser, P., Stotz, A. & Linder, P. (1993). A new yeast translation initiation factor suppresses a mutation in the eIF-4A RNA helicase. *EMBO J.* 12, 4005-11.

Costanzo, F., Campadelli-fiume, G., Foa-Tomasi, L. & Cassai, E. (1977). Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. *Journal of Virology* 21, 996-1001.

Courtney, R. J. & Benyesh-Melnick, M. (1974). Isolation and characterization of a large molecular-weight polypeptide of herpes simplex virus type 1. *Virology* 62, 539-51.

Crew, M. D. & Phanavanh, B. (2003). Exploiting virus stealth technology for xenotransplantation: reduced human T cell responses to porcine cells expressing herpes simplex virus ICP47. *Xenotransplantation* 10, 50-9.

Dahmus, M. E. (1996). Reversible phosphorylation of the C-terminal domain of RNA polymerase II. *J Biol Chem* 271, 19009-12.

de Bruyn Kops, A., Uprichard, S. L., Chen, M. & Knipe, D. M. (1998). Comparison of the intranuclear distributions of herpes simplex virus proteins involved in various viral functions. *Virology* 252, 162-78.

Deiss, L. P., Chou, J. & Frenkel, N. (1986). Functional domains within the α sequence involved in the cleavage-packaging of the herpes simplex virus DNA. *Journal of Virology* 59, 605-618.

Delius, H. & Clements, J. B. (1976). A partial denaturation map of herpes simplex virus type 1 DNA: evidence for inversion of the unique DNA regions. *J. Gen. Virol.* 33, 125-133.

DeLuca, N. A., McCarthy, A. M. & Schaffer, P. A. (1985). Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *J. of Virol.* 56, 558-570.

DeLuca, N. A. & Schaffer, P. A. (1985). Activation of immediate-early, early and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. *Mol. Cell. Biol.* 5, 1997-2008.

DeLuca, N. A. & Schaffer, P. A. (1987). Activities of herpes simplex virus type 1 (HSV-1) ICP4 gene specifying nonsense peptides. *Nucleic Acids Res.* 15, 4491-4511.

DeLuca, N. A. & Schaffer, P. A. (1988). Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. *Journal of Virology* 62, 732-743.

Dixon, R. A. F. & Schaffer, P. A. (1980). Fine structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. *J. of Virol.* 36, 189-203.

Doherty, A. J., Serpell, L. C. & Ponting, C. P. (1996). The helix-hairpin-helix DNA-binding motif: a structural basis for non-sequence-specific recognition of DNA. *Nucleic Acids Res.* 24, 2488-2497.

Dohner, K., Wolfstein, A., Prank, U., Echeverri, C., Dujardin, D., Vallee, R. & Sodeik, B. (2002). Function of dynein and dynactin in herpes simplex virus capsid transport. *Mol. Biol. Cell.* 13, 2795-809.

Dominguez, D., Altmann, M., Benz, J., Baumann, U. & Trachsel, H. (1999). Interaction of translation initiation factor eIF4G with eIF4A in the yeast Saccharomyces cerevisiae. *J Biol Chem* 274, 26720-6.

Dominguez, D., Kislig, E., Altmann, M. & Trachsel, H. (2001). Structural and functional similarities between the central eukaryotic initiation factor (eIF)4A-binding domain of mammalian eIF4G and the eIF4A-binding domain of yeast eIF4G. *Biochem J* 355, 223-30.

Du, M. X., Johnson, R. B., Sun, X. L., Staschke, K. A., Colacino, J. & Wang, Q. M. (2002). Comparative characterization of two DEAD-box RNA helicases in superfamily II: human translation-initiation factor 4A and hepatitis C virus non-structural protein 3 (NS3) helicase. *Biochem J* 363, 147-55.

Eidson, K. M., Hobbs, W. E., Manning, B. J., Carlson, P. & DeLuca, N. A. (2002). Expression of herpes simplex virus ICP0 inhibits the induction of interferon-stimulated genes by viral infection. *J Virol* 76, 2180-91.

Elgadi, M. M., Hayes, C. E. & Smiley, J. R. (1999). The herpes simplex virus vhs protein induces endoribonucleolytic cleavage of target RNAs in cell extracts. *J. Virol.* 73, 7153-64.

Elgadi, M. M. & Smiley, J. R. (1999). Picornavirus IRES elements target RNA cleavage events induced by the Herpes simplex virus vhs protein. *J. Virol.* 73, 9222-9231.

Ellison, K. S., Rice, S. A., Verity, R. & Smiley, J. R. (2000). Processing of alpha-globin and ICP0 mRNA in cells infected with herpes simplex virus type 1 ICP27 mutants. *J Virol* 74, 7307-19.

Emili, A. & Ingles, C. J. (1995). The RNA polymerase II carboxy-terminal domain: links to a bigger and better 'holoenzyme'? *Curr Opin Genet Dev* 5, 204-9.

Everett, R. D. (1984a). A detailed analysis of an HSV-1 early promoter: sequences involved in trans-activation by viral immediate-early products are not early gene specific. *Nuc. Acids Res.* 12, 3037-3056.

Everett, R. D. (1984b). Transactivation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. *EMBO J.* 3, 3135-3141.

Everett, R. D. (1987). The regulation of transcription of viral and cellular genes by herpesvirus immediate-early gene products. *Anticancer Res.* 7, 589-604.

Everett, R. D. (2000). ICP0 induces the accumulation of colocalizing conjugated ubiquitin. *J Virol* 74, 9994-10005.

Everett, R. D., Earnshaw, W. C., Findlay, J. & Lomonte, P. (1999a). Specific destruction of kinetochore protein CENP-C and disruption of cell division by herpes simplex virus immediate-early protein Vmw110. *EMBO J.* 18, 1526-38.

Everett, R. D., Freemont, P., Saitoh, H., Dasso, M., Orr, A., Kathoria, M. & Parkinson, J. (1998a). The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. *J. Virol.* 72, 6581-91.

Everett, R. D. & Maul, G. G. (1994). HSV-1 IE protein Vmw110 causes redistribution of PML. *EMBO J.* 13, 5062-5069.

Everett, R. D., Meredith, M. & Orr, A. (1999b). The ability of herpes simplex virus type 1 immediate-early protein Vmw110 to bind to a ubiquitin-specific protease contributes to its roles in the activation of gene expression and stimulation of virus replication. *J. Virol.* 73, 417-26.

Everett, R. D., Meredith, M., Orr, A., Cross, A., Kathoria, M. & Parkinson, J. (1997). A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. *EMBO J.* 16, 1519-30.

Everett, R. D., Orr, A. & Elliott, M. (1991a). High level expression and purification of herpes simplex virus type 1 immediate early polypeptide Vmw110. *Nucl. Acids Res.* 19, 6155-6161.

Everett, R. D., Orr, A. & Preston, C. M. (1998b). A viral activator of gene expression functions via the ubiquitin- proteasome pathway. *Embo J.* 17, 7161-9.

Everett, R. D., Paterson, T. & Elliott, M. (1990). The major transcriptional regulatory protein of herpes simplex type 1 includes a protease resistant DNA binding domain. *Nucleic Acids Research* 18, 4579.

Everett, R. D., Preston, C. M. & Stow, N. D. (1991b). Functional and genetic analysis of the role of Vmv 110 in herpes simplex virus replication. In *The control of herpes simplex virus gene expression*, pp. 50-76. Edited by E. K. Wagner. Boca Raton: CRC Press Inc.

Everett, R. D., Sourvinos, G. & Orr, A. (2003). Recruitment of herpes simplex virus type 1 transcriptional regulatory protein ICP4 into foci juxtaposed to ND10 in live, infected cells. *J Virol* 77, 3680-9.

Everly, D. N., Jr. & Read, G. S. (1997). Mutational analysis of the virion host shutoff gene (UL41) of herpes simplex virus (HSV): characterization of HSV type 1 (HSV-1)/HSV-2 chimeras. *J. Virol.* 71, 7157-66.

Everly, D. N. J., Feng, P., Mian, I. S. & Read, G. S. (2002). mRNA degradation by the virion host shutoff (Vhs) protein of herpes simplex virus: genetic and biochemical evidence that Vhs is a nuclease. *J. Virol.* 76, 8560-71.

Evstafieva, A. G., Beletsky, A. V., Borovjagin, A. V. & Bogdanov, A. A. (1993). Internal ribosome entry site of encephalomyocarditis virus RNA is unable to direct translation in Saccharomyces cerevisiae. *FEBS Lett.* 335, 273-6.

Faber, S. W. & Wilcox, K. W. (1986). Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences. *Nuc. Acids. Res.* 14, 6067-6083.

Farnsworth, A., Goldsmith, K. & Johnson, D. C. (2003). Herpes simplex virus glycoproteins gD and gE/gI serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm. *J Virol* 77, 8481-94.

Feng, P., Everly, D. N. J. & Read, G. S. (2001). mRNA decay during herpesvirus infections: interaction between a putative viral nuclease and a cellular translation factor. *J. Virol.* 75, 10272-80.

Fenwick, M. L. & Clark, J. (1982). Early and delayed shut-off of host protein synthesis in cells infected with herpes simplex virus. *J. Gen. Virol.* 61, 121-125.

Fenwick, M. L. & Everett, R. D. (1990a). Inactivation of the shutoff gene (UL41) of herpes simplex virus types 1 and 2. *J. Gen. Virol.* 71, 2961-2967.

Fenwick, M. L. & Everett, R. D. (1990b). Transfer of UL41, the gene controlling virion-associated host cell shutoff, between different strains of herpes simplex virus. *J. Gen. Virol.* 71, 411-418.

Fenwick, M. L. & McMenamin, M. M. (1984). Early virion-associated suppression of cellular protein synthesis by herpes simplex virus is accompanied by inactivation of mRNA. *J. Gen. VIrol.* 65, 1225-1228.

Fenwick, M. L. & Owen, S. A. (1988). On the control of immediate early (alpha) mRNA survival in cells infected with herpes simplex virus. *J. Gen. Virol.* 69, 2869-77.

Fenwick, M. L. & Walker, M. J. (1978). Suppression of the synthesis of cellular macromolecules by herpes simplex virus. *J. Gen. Virol.* 41, 37-51.

Ferrante, P., Mancuso, R., Pagani, E., Guerini, F. R., Calvo, M. G., Saresella, M., Speciale, L. & Caputo, D. (2000). Molecular evidences for a role of HSV-1 in multiple sclerosis clinical acute attack. *J Neurovirol.* 6, S109-14.

Flint, J. & Shenk, T. (1997). Viral transactivating proteins. *Annu. Rev. Genet.* 31, 177-212.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N. & Minson, T. (1992). Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J. Virol.* 66, 341-348.

Friedman, E., Katcher, A. H. & Brightman, V. J. (1977). Incidence of recurrent herpes labialis and upper respiratory infection: a prospective study of

the influence of biologic, social and psychologic predictors. *Oral Surg Oral Med Oral Pathol* 43, 873-8.

Frink, R. J., Anderson, K. P. & Wagner, E. W. (1981). Herpes simplex virus 1 Hind III fragment L encodes spliced and complementary mRNA species. *Journal of Virology* 39, 559-572.

Fruh, K., Ahn, K., Djaballah, H., Sempe, P., van Endert, P. M., Tampe, R., Peterson, P. A. & Yang, Y. (1995). A viral inhibitor of peptide transporters for antigen presentation. *Nature* 375, 415-8.

Fuchs, W., Klupp, B. G., Granzow, H., Hengartner, C., Brack, A., Mundt, A., Enquist, L. W. & Mettenleiter, T. C. (2002). Physical interaction between envelope glycoproteins E and M of pseudorabies virus and the major tegument protein UL49. *J Virol* 76, 8208-17.

Furukawa, L., Brevetti, L. S., Brady, S. E., Johnson, D., Ma, M., Welling, T. H. & Messina, L. M. (2000). Adenoviral-mediated gene transfer of ICP47 inhibits major histocompatibility complex class I expression on vascular cells in vitro. *J Vasc Surg* 31, 558-66.

Gaffney, D. F., McLauchlan, J., Whitton, J. L. & Clements, J. B. (1985). A modular system for assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate-early gene activation. *Nuc. Acids Res.* 13, 7847-7863.

Gallinari, P., Wiebauer, K., Nardi, M. C. & Jiricny, J. (1994). Localization of a 34-amino-acid segment implicated in dimerization of the herpes simplex virus type 1 ICP4 polypeptide by a dimerization trap. *J Virol* 68, 3809-20.

Gao, M. & Knipe, D. M. (1991). Potential role for herpes simplex virus ICP8 DNA replication protein in stimulation of late gene expression. *J Virol* 65, 2666-75.

Gelman, I. H. & Silverstein, S. (1985). Identification of immediate early genes from herpes simplex virus that transactivates the virus thymidine kinase gene. *Proc. Natl. Sci. USA* 82, 5265-5269.

Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J. & Spear, P. G. (1998). Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* 280, 1618-20.

Gerster, T. & Roeder, R. G. (1988). A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. *Proc. Natl. Acad. Sci. USA* 85, 6347-6351.

Gingras, A. C., Svitkin, Y., Belsham, G. J., Pause, A. & Sonenberg, N. (1996). Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus. *Proc Natl Acad Sci U S A* 93, 5578-83.

Glezen, W. P., Fernald, G. W. & Lohr, J. A. (1975). Acute respiratory disease of university students with special reference to the etiologic role of Herpesvirus hominis. *Am J Epidemiol* 101, 111-21.

Godowski, P. J. & Knipe, D. M. (1986). Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. *Proc. Natl. Sci. USA* 83, 256-260.

Goodkin, M. L., Ting, A. T. & Blaho, J. A. (2003). NF-kappaB is required for apoptosis prevention during herpes simplex virus type 1 infection. *J Virol* 77, 7261-80.

Goodrich, J. A., Hoey, T., Thut, C. J., Admon, A. & Tjian, R. (1993). Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell* 75, 519-530.

Greaves, R. & O'Hare, P. (1989). Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. *J. of Virol.* 63, 1641-1650.

Grondin, B. & DeLuca, N. (2000). Herpes simplex virus type 1 ICP4 promotes transcription preinitiation complex formation by enhancing the binding of TFIID to DNA. *J Virol* 74, 11504-10.

Grotzinger, T., Sternsdorf, T., Jensen, K. & Will, H. (1996). Interferon-modulated expression of genes encoding the nuclear-dot-associated proteins Sp100 and promyelocytic leukemia protein (PML). *Eur J Biochem* 238, 554-60.

Gu, B. & DeLuca, N. (1994). Requirements for activation of the herpes simplex virus glycoprotein C promoter in vitro by the viral regulatory protein ICP4. *J. Virol.* 68, 7953-7965.

Gu, B., Kuddus, R. & DeLuca, N. A. (1995). Repression of activator-mediated transcription by herpes simplex virus ICP4 via a mechanism involving interactions with the basal transcription factors TATA-binding protein and TFIIB. *Mol. Cell. Biol.* 15, 3618-3626.

Gu, B., Rivera-Gonzalez, R., Smith, C. A. & DeLuca, N. A. (1993). Herpes simplex virus infected cell polypeptide 4 preferentially represses sp1-activated over basal transcription from its own promoter. *Proc. Natl. Acad. Sci. USA* 90, 9528-9532.

Guichet, A., Copeland, J. W., Erdelyi, M., Hlousek, D., Zavorszky, P., Ho, J., Brown, S., Percival-Smith, A., Krause, H. M. & Ephrussi, A. (1997). The nuclear receptor homologue Ftz-F1 and the homeodomain protein Ftz are mutually dependent cofactors. *Nature* 385, 548-52.

Guldner, H. H., Szostecki, C., Grotzinger, T. & Will, H. (1992). IFN enhances expression of sp100, an autoantigen in primary biliary cirrhosis. *J. Immunol.* 149, 4067-4073.

Hann, L. E., Cook, W. J., Uprichard, S. L,, Knipe, D. M. & Coen, D. M. (1998). The role of herpes simplex virus ICP27 in the regulation of UL24 gene expression by differential polyadenylation. *J Virol* 72, 7709-14.

Hardwicke, M. A. & Sandri-Goldin, R. M. (1994). The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. *J. Virol.* 68, 4797-4810.

Hardy, W. R. & Sandri-Goldin, R. M. (1994). Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. *J. Virol.* 68, 7790-7799.

Harle, P., Sainz, B., Jr., Carr, D. J. & Halford, W. P. (2002). The immediate-early protein, ICP0, is essential for the resistance of herpes simplex virus to interferon-alpha/beta. *Virology* 293, 295-304.

Hayes, S. & O'Hare, P. (1993). Mapping of a major surface-exposed site in herpes simplex virus protein Vmw65 to a region of direct interaction in a transcriptional complex assembly. *Journal of Virology* 67, 852-862.

Hayward, G. S., Jacob, R. J., Wadsworth, S. C. & Roizman, B. (1975). Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. *Proc. Natl. Acad. Sci. USA* 72, 4243-4247.

Hellen, C. U. & Sarnow, P. (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes Dev.* 15, 1593-612.

Hengartner, C. J., Thompson, C. M., Zhang, J., Chao, D. M., Liao, S. M., Koleske, A. J., Okamura, S. & Young, R. A. (1995). Association of an activator with an RNA polymerase II holoenzyme. *Genes Dev.* 9, 897-910.

Herold, B. C., Visalli, R. J., Sumarski, N., Brandt, C. R. & Spear, P. G. (1994). Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. *J. Gen. Virol.* 75, 1211-1222.

Herold, B. C., WuDunn, D., Soltys, N. & Spear, P. G. (1991). Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J. Virol.* 65, 1090-1098.

Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H. & Johnson, D. (1995). Herpes simplex virus turns off the TAP to evade host immunity. *Nature* 375, 411-415.

Hoffman, M. A. & Palmenberg, A. C. (1995). Mutational analysis of the J-K stem-loop region of the encephalomyocarditis virus IRES. *J. Virol.* 69, 4399-406.

Homa, F. L. & Brown, J. C. (1997). Capsid assembly and DNA packaging in herpes simplex virus. *Rev Med Virol.* 7, 107-122.

Homa, F. L., Glorioso, J. C. & Levine, M. (1988). A specific 15-bp TATA box promoter element is required for expression of a herpes simplex virus type 1 late gene. *Genes & Development* 2, 40-53.

Homa, F. L., Otal, T. M., Glorioso, J. C. & Levine, M. (1986). Transcriptional control signals of a herpes simplex virus type 1 late (γ2) gene lie within the bases -34 to +126 relative to the 5' terminus of the mRNA. *Molecular Cellular Biology* 6, 3652-3666.

Honess, R. W. & Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J. Virol.* 14, 8-19.

Honess, R. W. & Roizman, B. (1975). Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. *Proc. Natl. Acad. Sci. USA* 72, 1276-1280.

Huang, C. J. & Wagner, E. K. (1994). The herpes simplex virus type 1 major capsid protein (VP5-UL19) promoter contains two cis-acting elements influencing late expression. *J Virol* 68, 5738-47.

Igarashi, K., Fawl, R., Roller, R. J. & Roizman, B. (1993). Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis. *J Virol* 67, 2123-32.

Imataka, H., Gradi, A. & Sonenberg, N. (1998). A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *Embo J* 17, 7480-9.

Imataka, H. & Sonenberg, N. (1997). Human eukaryotic translation initiation factor 4G (eIF4G) possesses two separate and independent binding sites for eIF4A. *Mol Cell Biol* 17, 6940-7.

Ingles, C. J., Shales, M., Cress, W. D., Triezenberg, S. J. & Greenblatt, J. (1991). Reduced binding of TFIID to transcriptionally compromised mutants of VP16. *Nature* 351, 588-590.

Ishov, A. M. & Maul, G. G. (1996). The periphery of nuclear domain 10 (ND10) as site of DNA virus deposition. *J Cell Biol* 134, 815-26.

Ishov, A. M., Sotnikov, A. G., Negorev, D., Vladimirova, O. V., Neff, N., Kamitani, T., Yeh, E. T., 3rd, S. J. F. & Maul, G. G. (1999). PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. *J. Cell. Biol.* 147, 221-34.

Jackson, S. A. & DeLuca, N. A. (2003). Relationship of herpes simplex virus genome configuration to productive and persistent infections. *Proc Natl Acad Sci U S A.* 100, 7871-6.

Jahedi, S., Markovitz, N. S., Filatov, F. & Roizman, B. (1999). Colocalization of the herpes simplex virus 1 UL4 protein with infected cell protein 22 in small, dense nuclear structures formed prior to onset of DNA synthesis. *J Virol* 73, 5132-8.

Jang, S. K., Krausslich, H. G., Nicklin, M. J., Duke, G. M., Palmenberg, A. C. & Wimmer, E. (1988). A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J Virol* 62, 2636-43.

Jean, S., LeVan, K. M., Song, B., Levine, M. & Knipe, D. M. (2001). Herpes simplex virus 1 ICP27 is required for transcription of two viral late (gamma 2) genes in infected cells. *Virology* 283, 273-84.

Jenkins, H. L. & Spencer, C. A. (2001). RNA polymerase II holoenzyme modifications accompany transcription reprogramming in herpes simplex virus type 1-infected cells. *J Virol* 75, 9872-84.

Joazeiro, C. A. & Weissman, A. M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 102, 549-52.

Johnson, D. C. & Huber, M. T. (2002). Directed egress of animal viruses promotes cell-to-cell spread. *J Virol* 76, 1-8.

Johnson, D. C., Wittels, M. & Spear, P. G. (1984). Binding to cells of virosomes containing herpes simplex virus type 1 glycoproteins and evidence for fusion. *J Virol* 52, 238-47.

Johnson, P. A. & Everett, R. D. (1986). The control of herpes simplex virus type 1 late gene transcription: a TATA-box/capsite region is sufficient for fully regulated activity. *Nuc. Acids Res.* 14, 8247-8264.

Jones, F. E., Smibert, C. A. & Smiley, J. R. (1995). Mutational analysis of the herpes simplex virus virion host shutoff protein: evidence that vhs functions in the absence of other viral proteins. *J. Virol.* 69, 4863-4871.

Jones, K. A. & Tjian, R. (1985). Sp1 binds to promoter sequences and activates herpes simplex virus "immediate-early" gene transcription *in vitro*. *Nature (London)* 317, 179-182.

Kaminski, A., Belsham, G. J. & Jackson, R. J. (1994). Translation of encephalomyocarditis virus RNA: parameters influencing the selection of the internal initiation site. *Embo J* 13, 1673-81.

Kaminski, A., Howell, M. T. & Jackson, R. J. (1990). Initiation of encephalomyocarditis virus RNA translation: the authentic initiation site is not selected by a scanning mechanism. *Embo J* 9, 3753-9.

Kaminski, A. & Jackson, R. J. (1998). The polypyrimidine tract binding protein (PTB) requirement for internal initiation of translation of cardiovirus RNAs is conditional rather than absolute. *RNA* 4, 626-38.

Karr, B. M. & Read, G. S. (1999). The virion host shutoff function of herpes simplex virus degrades the 5' end of a target mRNA before the 3' end. *Virology* 264, 195-204.

Katan, M. A., Haigh, C. P., Verrijzer, C. P., van der Vliet, P. C. & O'Hare, P. (1990). Characterization of a cellular factor which interacts functionally with Oct-1 in the assembly of a multicomponent transcription complex. *Nucleic Acid Research* 18, 6871-6880.

Kawaguchi, Y., Bruni, R. & Roizman, B. (1997a). Interaction of herpes simplex virus 1 α regulatory protein ICP0 with elongation factor 1δ: ICP0 affects translational machinery. *J. Virol.* 71, 1019-1024.

Kawaguchi, Y., Tanaka, M., Yokoymama, A., Matsuda, G., Kato, K., Kagawa, H., Hirai, K. & Roizman, B. (2001). Herpes simplex virus 1 alpha

regulatory protein ICP0 functionally interacts with cellular transcription factor BMAL1. *Proc Natl Acad Sci U S A.* 98, 1877-82.

Kawaguchi, Y., Van Sant, C. & Roizman, B. (1997b). Herpes simplex virus 1 alpha regulatory protein ICP0 interacts with and stabilizes the cell cycle regulator cyclin D3. *J. Virol.* 71, 7328-36.

Kibler, P. K., Duncan, J., Keith, B. D., Hupel, T. & Smiley, J. R. (1991). Regulation of herpes simplex virus true late gene expression: sequences downstream from the US11 TATA box inhibit expression from an unreplicated template. *J. Virol.* 65, 6749-6760.

Kim, D. B., Zabierowski, S. & DeLuca, N. A. (2002). The initiator element in a herpes simplex virus type 1 late-gene promoter enhances activation by ICP4, resulting in abundant late-gene expression. *J Virol* 76, 1548-58.

Kim, Y. K. & Jang, S. K. (1999). La protein is required for efficient translation driven by encephalomyocarditis virus internal ribosomal entry site. *J. Gen. Virol.* 80, 3159-66.

Klemm, R. D., Goodrich, J. A., Zhou, S. & Tjian, R. (1995). Molecular cloning and expression of the 32-kDa subunit of human TFIID reveals interactions with VP16 and TFIIB that mediate transcriptional activation. *Proc Natl Acad Sci U S A.* 92, 5788-92.

Knez, J., Bilan, P. T. & Capone, J. P. (2003). A single amino acid substitution in herpes simplex virus type 1 VP16 inhibits binding to the virion host shutoff protein and is incompatible with virus growth. *J. Virol.* 77, 2892-902.

Knipe, D. M., Batterson, W., Nosal, C., Roizman, B. & Buchan, A. (1981). Molecular genetics of herpes simplex virus. VI. Characterization of a temperature-sensitive mutant defective in the expression of all early viral gene products. *J Virol* 38, 539-47.

Kobayashi, N., Boyer, T. G. & Berk, A. J. (1995). A class of activation domains interacts directly with TFIIA and stimulates TFIIA-TFIID-promoter complex assembly. *Mol. Cell Biol.* 15, 6465-73.

Koffa, M. D., Clements, J. B., Izaurralde, E., Wadd, S., Wilson, S. A., Mattaj, I. W. & Kuersten, S. (2001). Herpes simplex virus ICP27 protein provides viral mRNAs with access to the cellular mRNA export pathway. *Embo J* 20, 5769-78.

Kolupaeva, V. G., Hellen, C. U. & Shatsky, I. N. (1996). Structural analysis of the interaction of the pyrimidine tract-binding protein with the internal ribosomal entry site of encephalomyocarditis virus and foot-and-mouth disease virus RNAs. *RNA* 2, 1199-212.

Kolupaeva, V. G., Lomakin, I. B., Pestova, T. V. & Hellen, C. U. (2003). Eukaryotic initiation factors 4G and 4A mediate conformational changes downstream of the initiation codon of the encephalomyocarditis virus internal ribosomal entry site. *Mol. Cell Biol.* 23, 687-98.

Kolupaeva, V. G., Pestova, T. V., Hellen, C. U. & Shatsky, I. N. (1998). Translation eukaryotic initiation factor 4G recognizes a specific structural element within the internal ribosome entry site of encephalomyocarditis virus RNA. *J Biol Chem* 273, 18599-604.

Krikorian, C. R. & Read, G. S. (1991). In vitro mRNA degradation system to study the virion host shutoff function of herpes simplex virus. *J. Virol.* 65, 112-122.

Kristensson, K., Lycke, E., Roytta, M., Svennerholm, B. & Vahlne, A. (1986). Neuritic transport of herpes simplex virus in rat sensory neurons in vitro. Effects of substances interacting with microtubular function and axonal flow [nocodazole, taxol and erythro-9-3-(2-hydroxynonyl)adenine]. *J. Gen. Virol.* 67, 2023-8.

Kristie, T. M., Lebowitz, J. H. & Sharp, P. A. (1989). The octamer-binding proteins form multi-protein-DNA complexes with the HSV alpha TIF regulatory protein. *EMBO Journal* 8, 4229-4238.

Kristie, T. M. & Roizman, B. (1984). Separation of sequences defining basal expression from those conferring alpha gene recognition within the

regulatory domains of herpes simplex virus alpha genes. *Proc. Natl. Acad. Science. USA* 84, 71-75.

Kristie, T. M. & Roizman, B. (1987). Host cell proteins binds to the cis-acting site required for virion-mediated induction of herpes simplex virus alpha genes. *Proc. Natl. Acad. Sci. USA* 84, 71-75.

Kristie, T. M. & Sharp, P. A. (1990). Interaction of the Oct-1 POU subdomains with specific DNA sequences and with the HSV alpha-trans-activator protein. *Genes Dev.* 4, 2383-2396.

Kristie, T. M. & Sharp, P. A. (1993). Purification of the cellular C1 factor required for the stable recognition of the Oct-1 homeodomain by the herpes simplex virus α-trans-induction factor (VP16). *Journal of Biological Chemistry* 268, 6525-6534.

Kuddus, R., Gu, B. & DeLuca, N. A. (1995). Relationship between TATA-binding protein and herpes simplex virus type 1 ICP4 DNA-binding sites in complex formation and repression of transcription. *J. Virol.* 69, 5568-5575.

Kwong, A. D. & Frenkel, N. (1987). Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. *Proc. Natl. Acad. Sci. USA* 84, 1926-1930.

Kwong, A. D., Kruper, J. A. & Frenkel, N. (1988). Herpes simplex virus virion host shutoff function. *J. Virol.* 62, 912-921.

La Boissiere, S., T., H. & O'Hare, P. (1999). HCF-dependent nuclear import of VP16. *EMBO J.* 18, 480-9.

Lam, Q., Smibert, C. A., Koop, K. E., Lavery, C., Capone, J. P., Weinheimer, S. P. & Smiley, J. R. (1996). Herpes simplex virus VP16 rescues viral mRNAs from destruction by the virion host shutoff function. *EMBO J.* 15, 2575-2581.

LaMarco, K. L. & McKnight, S. L. (1989). Purification of a set of cellular polypeptides that bind to the purine-rich cis-regulatory element of herpes simplex virus immediate early genes. *Genes Dev.* 3, 1372-83.

Lamberti, C. & Weller, S. K. (1998). The herpes simplex virus type 1 cleavage/packaging protein, UL32, is involved in efficient localization of capsids to replication compartments. *J Virol* 72, 2463-73.

Lamphear, B. J., Kirchweger, R., Skern, T. & Rhoads, R. E. (1995). Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. *J. Biol. Chem.* 270, 21975-83.

Lavau, C., Marchio, A., Fagioli, M., Jansen, J., Falini, B., Lebon, P., Grosveld, F., Pandolfi, P. P., Pelicci, P. G. & Dejean, A. (1995). The acute promyelocytic leukaemia-associated PML gene is induced by interferon. *Oncogene* 11, 871-6.

Leib, D. A., Harrison, T. E., Laslo, K. M., Machalek, M. A., Moorman, N. J. & Virgin, H. W. (1999). Interferons Regulate the Phenotype of Wild-type and Mutant Herpes Simplex Viruses In Vivo. *J. Exp. Med.* 189, 663-672.

Lengyel, J., Guy, C., Leong, V., Borge, S. & Rice, S. A. (2002). Mapping of functional regions in the amino-terminal portion of the herpes simplex virus ICP27 regulatory protein: importance of the leucine-rich nuclear export signal and RGG Box RNA-binding domain. *J Virol* 76, 11866-79.

Leopardi, R., Ward, P. L., Ogle, W. O. & Roizman, B. (1997). Association of herpes simplex virus regulatory protein ICP22 with transcriptional complexes containing EAP, ICP4, RNA polymerase II, and viral DNA requires posttranslational modification by the U_L13 protein kinase. *J. Virol.* 71, 1133-1139.

Lieu, P. T. & Wagner, E. K. (2000). Two leaky-late HSV-1 promoters differ significantly in structural architecture. *Virology* 272, 191-203.

Ligas, M. W. & Johnson, D. C. (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. *Journal of VIrology* 62, 1486-1494.

Lin, Y.-S. & Green, M. R. (1991). Mechanism of action of an acidic transcriptional activator *in vitro*. *Cell* 64, 971-981.

Lin, Y.-S., Ha, I., Maldonado, D., Reinberg, D. & Green, M. (1991). Binding of general transcription factor TFIIB to an acidic activating region. *Nature* 353, 569-571.

Lindberg, A. & Kreivi, J. P. (2002). Splicing inhibition at the level of spliceosome assembly in the presence of herpes simplex virus protein ICP27. *Virology* 294, 189-98.

Livingstone-Zatchej, M., Meier, A., Suter, B. & Thoma, F. (1997). RNA polymerase II transcription inhibits DNA repair by photolyase in the transcribed strand of active yeast genes. *Nucleic Acids Res* 25, 3795-800.

Lomakin, I. B., Hellen, C. U. & Pestova, T. V. (2000). Physical association of eukaryotic initiation factor 4G (eIF4G) with eIF4A strongly enhances binding of eIF4G to the internal ribosomal entry site of encephalomyocarditis virus and is required for internal initiation of translation. *Mol. Cell Biol.* 20, 6019-29.

Lomonte, P., Sullivan, K. F. & Everett, R. D. (2001). Degradation of nucleosome-associated centromeric histone H3-like protein CENP-A induced by herpes simplex virus type 1 protein ICP0. *J. Biol. Chem.* 276, 5829-35.

Long, M. C., Leong, V., Schaffer, P. A., Spencer, C. A. & Rice, S. A. (1999). ICP22 and the UL13 protein kinase are both required for herpes simplex virus-induced modification of the large subunit of RNA polymerase II [In Process Citation]. *J Virol* 73, 5593-604.

Longnecker, R. & Roizman, B. (1986). Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction *a* sequences, an origin of DNA synthesis, and several genes including those specifying glyocoprotein E and the α47 gene. *Journal of Virology* 58, 583-591.

Loomis, J. S., Bowzard, J. B., Courtney, R. J. & Wills, J. W. (2001). Intracellular trafficking of the UL11 tegument protein of herpes simplex virus type 1. *J Virol* 75, 12209-19.

Lopez, P., Van Sant, C. & Roizman, B. (2001). Requirements for the nuclear-cytoplasmic translocation of infected-cell protein 0 of herpes simplex virus 1. *J. Virol.* 75, 3832-40.

Lu, H., Flores, O., Weinmann, R. & Reinberg, D. (1991). The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. *Proc Natl Acad Sci U S A* 88, 10004-8.

Lu, P., Jones, F. E., Saffran, H. A. & Smiley, J. R. (2001a). The Herpes Simples Virus virion host shutoff protein requires a mammalian factor for efficient *in vitro* endoribonuclease activity. *J. Virol.* 75, 1172-85.

Lu, P., Saffran, H. A. & Smiley, J. R. (2001b). The vhs1 mutant form of the Herpes Simplex Virus virion host shutoff protein retains significant internal ribosome entry site-directed RNA cleavage activity. *J Virol.* 75, 1072-6.

Luciano, R. L. & Wilson, A. C. (2002). An activation domain in the C-terminal subunit of HCF-1 is important for transactivation by VP16 and LZIP. *Proc Natl Acad Sci U S A.* 99, 13403-8.

Mackem, S. & Roizman, B. (1982a). Differentiation between α promoter and regulatory regions of herpes simplex virus 1: the functional domains and sequence of a moveable α regulator. *Proc. Natl. Acad. Sci. USA* 79, 4917-4921.

Mackem, S. & Roizman, B. (1982b). Structural features of the herpes simplex virus alpha 4, 0, and 27 promoter-regulatory sequences which confer alpha regulation on chimeric thymidine kinase genes. *Journal of Virology* 44, 939-949.

Mader, S., Lee, H., Pause, A. & Sonenberg, N. (1995). The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. *Mol Cell Biol* 15, 4990-7.

Maniatis, T., Fritsch, E. R. & Sambrook, J. (1989). Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor, New York: Cold Spring Habor Laboratory Publications.

Markovitz, N. S. & Roizman, B. (2000). Small dense nuclear bodies are the site of localization of herpes simplex virus 1 U(L)3 and U(L)4 proteins and of ICP22 only when the latter protein is present. *J Virol* 74, 523-8.

Martinez-Salas, E., Ramos, R., Lafuente, E. & Lopez de Quinto, S. (2001). Functional interactions in internal translation initiation directed by viral and cellular IRES elements. *J Gen Virol* 82, 973-84.

Maul, G. G. & Everett, R. D. (1994). The nuclear location of PML, a cellular member of the C_3HC_4 zinc-binding domain protein family, is rearranged during herpes simplex virus infection by he C_3HC_4 viral protein ICP0. *J. Gen. Viral.* 75, 1223-1233.

Maul, G. G., Guldner, H. H. & Spivack, J. G. (1993). Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0). *J. Gen. Virol.* 74, 2679-2690.

Mavromara-Nazos, P. & Roizman, B. (1989). Delineation of regulatory domains of early (β) and late (γ2) genes by construction of chimeric genes expressed in herpes simplex virus 1 genomes. *Proceedings of the National Academy of Sciences USA* 86, 4071-4075.

McCarthy, A. M., McMahan, L. & Schaffer, P. A. (1989). Herpes simplex virus type 1 ICP27 deletion mutant exhibits altered patterns of transcription and are DNA deficient. *J. Virol.* 63, 18-27.

McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E. & Taylor, P. (1988). The complete sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* 69., 1531-1574.

McGeoch, D. J., Dolan, A., Donald, S. & Rixon, F. J. (1985). Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. *Journal of Molecular Biology* 181, 1-13.

McGregor, F., Phelan, A., Dunlop, J. & Clements, J. B. (1996). Regulation of herpes simplex virus poly(A) site usage and the action of immediate-early protein IE63 in the early-late switch. *J. Virol.* 70, 1931-1940.

McKnight, J. C. L., Kristie, T. M. & Roizman, B. (1987). Binding of the virion protein mediating alpha gene induction in herpes simplex virus 1-infected cells to its cis site requires cellular proteins. *Proc. Natl. Acad. Sci. USA* 84, 2061-7065.

McLauchlan, J., Addison, C., Craigie, M. C. & Rixon, F. J. (1992). Noninfectious l-particles supply functions which can facilitate infection by HSV-1. *Virology* 190, 682-688.

McMillan, J. A., Weiner, L. B., Higgins, A. M. & Lamparella, V. J. (1993). Pharyngitis associated with herpes simplex virus in college students. *Pediatr Infect Dis J* 12, 280-4.

McNabb, D. S. & Courtney, R. J. (1992). Characterization of the large tegument protein (ICP1/2) of herpes simplex virus type 1. *Virology* 190, 221-232.

Mears, W. & Rice, S. A. (1996). The RGG box motif of the herpes simplex virus ICP27 protein mediates an RNA -binding activity and determines in vivo methylation. *J. Virol.* 70, 7445-7453.

Mears, W. E., Lam, V. & Rice, S. A. (1995). Identification of nuclear and nucleolar localization signals in the herpes simplex virus regulatory protein ICP27. *J Virol* 69, 935-47.

Mears, W. E. & Rice, S. A. (1998). The herpes simplex virus immediate-early protein ICP27 shuttles between nucleus and cytoplasm. *Virology* 242, 128-37.

Methot, N., Pause, A., Hershey, J. W. & Sonenberg, N. (1994). The translation initiation factor eIF-4B contains an RNA-binding region that is distinct and independent from its ribonucleoprotein consensus sequence. *Mol Cell Biol* 14, 2307-16.

Methot, N., Pickett, G., Keene, J. D. & Sonenberg, N. (1996a). In vitro RNA selection identifies RNA ligands that specifically bind to eukaryotic translation initiation factor 4B: the role of the RNA remotif. *Rna* 2, 38-50.

Methot, N., Song, M. S. & Sonenberg, N. (1996b). A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. *Mol. Cell Biol.* 16, 5328-34.

Mettenleiter, T. C. (2002). Herpesvirus assembly and egress. *J Virol* 76, 1537-47.

Metzler, D. W. & Wilcox, K. W. (1985). Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. *Journal of Virology* 55, 329-337.

Mitchell, C., Blaho, J. A., McCormick, A. L. & Roizman, B. (1997). The nucleotidylylation of herpes simplex virus 1 regulatory protein alpha22 by human casein kinase II. *J Biol Chem* 272, 25394-400.

Montgomery, R. I., Warner, M. S., Lum, B. J. & Spear, P. G. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* 87, 427-436.

Morino, S., Imataka, H., Svitkin, Y. V., Pestova, T. V. & Sonenberg, N. (2000). Eukaryotic translation initiation factor 4E (eIF4E) binding site and the middle one-third of eIF4GI constitute the core domain for cap-dependent translation, and the C-terminal one-third functions as a modulatory region. *Mol Cell Biol* 20, 468-77.

Mossman, K. L., Macgregor, P. F., Rozmus, J. J., Goryachev, A. B., Edwards, A. M. & Smiley, J. R. (2001). Herpes simplex virus triggers and then disarms a host antiviral response. *J Virol* 75, 750-8.

Mossman, K. L. & Smiley, J. R. (2002). Herpes simplex virus ICP0 and ICP34.5 counteract distinct interferon-induced barriers to virus replication. *J Virol* 76, 1995-8.

Mullen, M.-A., Gersterger, S., Ciufo, D. M., Mosca, J. D. & Hayward, G. S. (1995). Evaluation of colocalization interactions between the IE110, IE175, and IE63 transactivator proteins of herpes simplex virus within subcellular punctate structures. *J. Virol.* 69, 476-491.

Muller, M. T. (1987). Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. *J. Virol.* 61, 858-865.

Muller, S. & Dejean, A. (1999). Viral immediate-early proteins abrogate the modification by SUMO-1 of PML and Sp100 proteins, correlating with nuclear body disruption. *J. Virol.* 73, 5137-43.

Murphy, J. A., Duerst, R. J., Smith, T. J. & Morrison, L. A. (2003). Herpes simplex virus type 2 virion host shutoff protein regulates alpha/beta interferon but not adaptive immune responses during primary infection in vivo. *J Virol* 77, 9337-45.

Nalwanga, D., Rempel, S., Roizman, B. & Baines, J. D. (1996). The UL 16 gene product of herpes simplex virus 1 is a virion protein that colocalizes with intranuclear capsid proteins. *Virology* 226, 236-42.

Neff, C. L. & Sachs, A. B. (1999). Eukaryotic translation initiation factors 4G and 4A from Saccharomyces cerevisiae interact physically and functionally. *Mol Cell Biol* 19, 5557-64.

Ng, T. I., Chang, Y. E. & Roizman, B. (1997). Infected cell protein 22 of herpes simplex virus 1 regulates the expression of virion host shutoff gene U(L)41. *Virology* 234, 226-34.

Nicholl, M. J., Robinson, L. H. & Preston, C. M. (2000). Activation of cellular interferon-responsive genes after infection of human cells with herpes simplex virus type 1. *J Gen Virol* 81, 2215-8.

Nishioka, Y. & Silverstein, S. (1977). Degradation of cellular mRNA during infection by herpes simplex virus. *Proc. Natl. Acad. Sci. USA* 74, 2370-2374.

Nishioka, Y. & Silverstein, S. (1978). Requirement of protein synthesis for the degradation of host mRNA in Friend erythroleukemia cells infected with HSV-2. *J. Virol.* 27, 619-627.

O'Hare, P., Goding, C. R. & Haigh, A. (1988). Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular

octamer-binding factor mediates specific induction of virus immediate-early gene expression. *EMBO J.* 7, 4231-4238.

O'Hare, P. & Gooding, C. R. (1988). Herpes simplex virus regulatory elements and the immunoglobin octamer domain a binds a common factor and are both targets for virion transactivation. *Cell* 52, 435-445.

O'Hare, P. & Hayward, G. S. (1985a). Evidence for a direct role for both the 175,000- and 110,000- molecular-weight immediate-early proteins of herpes simplex virus in the transcativation of delayed-early promoter. *J. Virol.* 53, 751-760.

O'Hare, P. & Hayward, G. S. (1985b). Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. *J. Virol.* 56, 723-733.

O'Toole, J. M., Aubert, M., Kotsakis, A. & Blaho, J. A. (2003). Mutation of the protein tyrosine kinase consensus site in the herpes simplex virus 1 alpha22 gene alters ICP22 posttranslational modification. *Virology* 305, 153-67.

Ogle, W. O. & Roizman, B. (1999). Functional anatomy of herpes simplex virus 1 overlapping genes encoding infected-cell protein 22 and US1.5 protein. *J Virol* 73, 4305-15.

Ojala, P. M., Sodeik, B., Ebersold, M. W., Kutay, U. & Helenius, A. (2000). Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro. *Mol. Cell. Biol.* 20, 4922-31.

Oroskar, A. A. & Read, G. S. (1987). A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-early (alpha) mRNAs. *J. Virol.* 61, 604-606.

Oroskar, A. A. & Read, G. S. (1989). Control of mRNA stability by the virion host shutoff function of herpes simplex virus. *J. Virol.* 63, 1897-1906.

Osborne, L. R., Martindale, D., Scherer, S. W., Shi, X. M., Huizenga, J., Heng, H. H., Costa, T., Pober, B., Lew, L., Brinkman, J., Rommens, J., Koop, B.

& Tsui, L. C. (1996). Identification of genes from a 500-kb region at 7q11.23 that is commonly deleted in Williams syndrome patients. *Genomics* 36, 328-36.

Overton, H., McMillan, D., Hope, L. & Wong-Kai-In, P. (1994). Production of host shutoff-defective mutants of herpes simplex virus type 1 by inactivation of the UL13 gene. *Virology* 202, 97-106.

Pak, A. S., Everly, D. N., Knight, K. & Read, G. S. (1995). The virion host shutoff protein of herpes simplex virus inhibits reporter gene expression in the absence of other viral gene products. *Virology* 211, 491-506.

Panagiotidis, C. A., Lium, E. K. & Silverstein, S. J. (1997). Physical and functional interactions between herpes simplex virus immediate-early proteins ICP4 and ICP27. *J. Virol.* 71, 1547-1557.

Parkinson, J. & Everett, R. D. (2000). Alphaherpesvirus proteins related to herpes simplex virus type 1 ICP0 affect cellular structures and proteins. *J. Virol.* 74, 10006-17.

Parkinson, J., Lees-Miller, S. P. & Everett, R. D. (1999). Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA- dependent protein kinase. *J Virol* 73, 650-7.

Paterson, T. & Everett, R. D. (1988a). Mutational dissection of the HSV-1 immediate-early protein Vmw175 involved in transcriptional transactivation and repression. *Virology* 166, 186-196.

Paterson, T. & Everett, R. D. (1988b). The regions of the herpes simplex virus type 1 immediate early protein Vme175 required for site specific DNA binding closely correspond to those involved in transcriptional regulation. *Nucl. Acids Res.* 16, 11005-11025.

Payne, J. M., Laybourn, P. J. & Dahmus, M. E. (1989). The transition of RNA polymerase II from initiation to elongation is associated with phosphorylation of the carboxyl-terminal domain of subunit IIa. *J. Biol. Chem.* 264, 19621-19629.

Pelletier, J., Kaplan, G., Racaniello, V. R. & Sonenberg, N. (1988). Cap-independent translation of poliovirus mRNA is conferred by sequence elements within the 5' noncoding region. *Mol Cell Biol* 8, 1103-12.

Pestova, T. V., Hellen, C. U. & Shatsky, I. N. (1996a). Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. *Mol Cell Biol* 16, 6859-69.

Pestova, T. V., Kolupaeva, V. G., Lomakin, I. B., Pilipenko, E. V., Shatsky, I. N., Agol, V. I. & Hellen, C. U. (2001). Molecular mechanisms of translation initiation in eukaryotes. *Proc Natl Acad Sci U S A* 98, 7029-36.

Pestova, T. V., Shatsky, I. N., Fletcher, S. P., Jackson, R. J. & Hellen, C. U. (1998). A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. *Genes Dev* 12, 67-83.

Pestova, T. V., Shatsky, I. N. & Hellen, C. U. (1996b). Functional dissection of eukaryotic initiation factor 4F: the 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. *Mol. Cell Biol.* 16, 6870-8.

Phelan, A., Carmo-Fonseca, M., McLaughlan, J., Lamond, A. I. & Clements, J. B. (1993). A herpes simplex virus type 1 IE gene product, IE63, regulate small nuclear ribonculeoprotein distribution. *Proc. Natl. Sci. USA* 90, 9056-9060.

Phelan, A. & Clements, J. B. (1997). Herpes simplex virus type 1 immediate early protein IE63 shuttles between nuclear compartments and the cytoplasm. *J. Gen. Virol.* 78, 3327-3331.

Phelan, A., Dunlop, J., Patel, A. H., Stow, N. D. & Clements, J. B. (1997). Nuclear sites of herpes simplex virus type 1 DNA replication and transcripton colocalize at early times postinfection and are largely distinct from RNA processing factors. *J. Virol.* 71, 1124-1132.

Poffenberger, K. L., Raichlen, P. E. & Herman, R. C. (1993). *In vitro* characterization of a herpes simplex virus type 1 ICP22 deletion mutant. *Virus Genes* 7, 171-186.

Polvino-Bodnar, M., Orberg, P. K. & Schaffer, P. A. (1987). Herpes simplex virus type 1 oriL is not required for virus replication or for the establishment and reactivation of latent infection in mice. *J Virol* 61, 3528-35.

Pomerantz, J. L., Kristie, T. M. & Sharp, P. A. (1992). Recognition of the surface of a homeo domain protein. *Genes Dev.* 6, 2047-57.

Post, L. E., Conley, A. J., Mocarski, E. S. & Roizman, B. (1981). Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. *Cell* 24, 555-565.

Post, L. E. & Roizman, B. (1981). A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. *Cell* 25, 27-232.

Powell, K. L. & Courtney, R. J. (1975). Polypeptide synthesized in herpes simplex virus type 2-infected HEp-2 cells. *Virology* 66, 217-28.

Prat, A., Schmid, S. R., Buser, P., Blum, S., Trachsel, H., Nielsen, P. J. & Linder, P. (1990). Expression of translation initiation factor 4A from yeast and mouse in Saccharomyces cerevisiae. *Biochim Biophys Acta* 1050, 140-5.

Preston, C. M. (1979). Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK. *Journal of Virology* 29, 275-284.

Preston, C. M., Frame, M. C. & Campbell, M. E. M. (1988). A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate-early gene regulatory DNA sequence. *Cell* 52, 425-434.

Preston, V. G., al-Kobaisi, M. F., McDougall, I. M. & Rixon, F. J. (1994). The herpes simplex virus gene UL26 proteinase in the presence of the UL26.5 gene product promotes the formation of scaffold-like structures. *J Gen Virol* 75, 2355-66.

Ptak, C., Prendergast, J. A., Hodgins, R., Kay, C. M., Chau, V. & Ellison, M. J. (1994). Functional and physical characterization of the cell cycle ubiquitin-conjugating enzyme CDC34 (UBC3). Identification of a functional determinant within the tail that facilitates CDC34 self-association. *J Biol Chem* 269, 26539-45.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M. & Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* 24, 218-29.

Purves, F. C., Ogle, W. O. & Roizman, B. (1993). Processing of the herpes simplex virus regulatory protein α22 mediated by the UL13 protein kinase determines the accumulation of a subset of α and γ mRNAs and proteins in infected cells. *Proc. Natl. Sci. USA* 90, 6701-6705.

Purves, F. C. & Roizman, B. (1992). The UL13 gene of herpes simplex virus 1 encodes the functions for the posttranslational processing associated with phosphorylation of the regulatory protein α22. *Proc. Natl. Sci. USA* 89, 7310-7314.

Quinlan, M. P., Chen, L. B. & Kniipe, D. M. (1984). The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. *Cell* 36, 857-868.

Quinlan, M. P. & Knipe, D. M. (1985). Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. *Mol. Cell. Biol.* 5, 957-963.

Read, G. S., Bradley, B. M. & Knight, K. (1993). Isolation of a herpes simplex virus type 1 mutant with a deletion in the virion host shutoff gene and identification of multiple forms o fthe vhs (UL41) polypeptides. *J. Virol.* 67, 7149-7160.

Read, G. S. & N., F. (1983). Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptide synthesis and exhibiting

abnormal synthesis of a (immediate early) viral polypeptides. *J. Virol.* 46, 498-512.

Reynolds, A. E., Ryckman, B. J., Baines, J. D., Zhou, Y., Liang, L. & Roller, R. J. (2001). U(L)31 and U(L)34 proteins of herpes simplex virus type 1 form a complex that accumulates at the nuclear rim and is required for envelopment of nucleocapsids. *J Virol* 75, 8803-17.

Reynolds, A. E., Wills, E. G., Roller, R. J., Ryckman, B. J. & Baines, J. D. (2002). Ultrastructural localization of the herpes simplex virus type 1 UL31, UL34, and US3 proteins suggests specific roles in primary envelopment and egress of nucleocapsids. *J Virol* 76, 8939-52.

Rice, S. A. & Knipe, D. M. (1990). Genetic evidence for two distinct transactivation functions of the herpes simplex virus α protein ICP27. *J. Virol.* 64, 1704-1715.

Rice, S. A., Lam, V. & Knipe, D. M. (1993). The acidid amino-terminal region of herpes simplex virus type 1 alpha protein ICP27 is required for an essential lytic function. *J. Virol.* 67, 1778-1787.

Rice, S. A., Long, M. C., Lam, V., Schaffer, P. A. & Spencer, C. A. (1995). Herpes simplex virus immediate-early protein ICP22 is required for viral modification of host RNA polymerase II and establishment of the normal viral transcription program. *J. Virol.* 69, 5550-5559.

Rice, S. A., Long, M. C., Lam, V. & Spencer, C. A. (1994). RNA polymerase II is aberrantly phosophorylated and localized to viral replication compartments following herpes simplex virus infection. *J. Virol.* 68, 988-1001.

Richter, N. J., Rogers, G. W., Jr., Hensold, J. O. & Merrick, W. C. (1999). Further biochemical and kinetic characterization of human eukaryotic initiation factor 4H. *J. Biol. Chem.* 274, 35415-35424.

Richter-Cook, N. J., Dever, T. E., Hensold, J. O. & Merrick, W. C. (1998). Purification and characterization of a new eukaryotic protein translation factor. Eukaryotic initiation factor 4H. *J. Biol. Chem.* 273, 7579-87.

Rivera-Gonzalez, R. A., Imbalzano, A. N., Gu, B. & DeLuca, N. A. (1994). The role of ICP4 repressor activity in temporal expression of the IE-3 and latency assosciated transcript promoters during HSV-1 infection. *Virology* 202, 550-564.

Roberts, M. S., Boundy, A., O'Hare, P., Pizzorno, M. C., Ciufo, D. M. & Hayward, G. S. (1988). Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (α4) promoter and a specific binding site for the IE175 (ICP4) protein. *J. Virol.* 62, 4307-4320.

Rogers, G. W., Jr., Komar, A. A. & Merrick, W. C. (2002). eIF4A: the godfather of the DEAD box helicases. *Prog Nucleic Acid Res Mol Biol* 72, 307-31.

Rogers, G. W., Jr., Richter, N. J. & Merrick, W. C. (1999). Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. *J. Biol. Chem.* 274, 12236-44.

Rogers, G. W. J., Richter, N. J., Lima, W. F. & Merrick, W. C. (2001). Modulation of the helicase activity of eIF4A by eIF4B, eIF4H, and eIF4F. *J. Biol. Chem.* 276, 30914-22.

Roizman, B. (1996). Herpesviridae. In *Fields Virology*, 3rd edn, pp. 2221-2230. Edited by B. N. Fields, D. M. Knipe, P. M. Howley & e. al. Philidelphia: Lippincott-Raven Pubilshers.

Roizman, B., Borman, G. S. & Rousta, M. K. (1965). Macromolecular synthesis in cells infected with herpes simplex virus. *Nature* 206, 1374-5.

Roizman, B. & Knipe, D. M. (2001). Herpes simplex viruses and their replication. In *Fields Virology*, 4th edn, pp. 2399-2459. Edited by D. M. Knipe & P. H. Howley: Lippincott Williams and Wilkins.

Roller, R. J., Zhou, Y., Schnetzer, R., Ferguson, J. & DeSalvo, D. (2000). Herpes simplex virus type 1 U(L)34 gene product is required for viral envelopment. *J Virol* 74, 117-29.

Sacks, W. R., Greene, C. C., Aschman, D. P. & Schaffer, P. A. (1985). Herpes simplex virus type 1 ICP27 is an essential regulatory protein. *J. Virol.* 55, 796-805.

Sacks, W. R. & Schaffer, P. A. (1987). Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICPO exhibit impaired growth in cell culture. *J. Virol.* 61, 829-839.

Samady, L., Costigliola, E., MacCormac, L., McGrath, Y., Cleverley, S., Lilley, C. E., Smith, J., Latchman, D. S., Chain, B. & Coffin, R. S. (2003). Deletion of the virion host shutoff protein (vhs) from herpes simplex virus (HSV) relieves the viral block to dendritic cell activation: potential of vhs- HSV vectors for dendritic cell-mediated immunotherapy. *J. Virol.* 77, 3768-76.

Samaniego, L. A., Webb, A. L. & DeLuca, N. A. (1995). Functional interactions between herpes simplex virus immediate-early proteins during infection: gene expression as a consequence of ICP27 and different domains of ICP4. *J. Virol.* 69, 5705-5715.

Sandri-Goldin, R. M. (1998). ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. *Genes Dev.* 12, 868-879.

Sandri-Goldin, R. M. & Hibbard, M. K. (1996). The herpes simplex virus type 1 regulatory protein ICP27 coimmunoprecipitates with anti-sm antiserum, and the C terminus appears to be required for this interaction. *J. Virol.* 70, 108-118.

Sandri-Goldin, R. M., Hibbard, M. K. & Hardwicke, M. A. (1995). The C-terminal repressor region of herpes simplex virus type 1 ICP27 is required for the redistribution of small nuclear ribonucleoprotein particles and splicing factor SC35; however, these alterations are not sufficient to inhibit host cell splicing. *J. Virol.* 69, 6063-6076.

Sarmiento, M., Haffey, M. & Spear, P. G. (1979). Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B2) in virion infectivity. *J. Virol.* 29, 1149-58.

Scaria, A., Sullivan, J. A., St George, J. A., Kaplan, J. M., Lukason, M. J., Morris, J. E., Plog, M., Nicolette, C., Gregory, R. J. & Wadsworth, S. C. (2000). Adenoviral vector expressing ICP47 inhibits adenovirus-specific cytotoxic T lymphocytes in nonhuman primates. *Mol Ther* 2, 505-14.

Schek, N. & Bachenheimer, S. L. (1985). Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of Vero cells. *J. Virol.* 55, 601-610.

Schultz, M. C., Choe, S. Y. & Reeder, R. H. (1991). Specific initiation by RNA polymerase I in a whole-cell extract from yeast. *Proc. Natl. Acad. Sci. U S A* 88, 1004-8.

Schultz, M. C., Hockman, D. J., Harkness, T. A., Garinther, W. I. & Altheim, B. A. (1997). Chromatin assembly in a yeast whole-cell extract. *Proc. Natl. Acad. Sci. U S A* 94, 9034-9.

Sciabica, K. S., Dai, Q. J. & Sandri-Goldin, R. M. (2003). ICP27 interacts with SRPK1 to mediate HSV splicing inhibition by altering SR protein phosphorylation. *Embo J* 22, 1608-19.

Sears, A. E., Halliburton, I. W., Meignier, B., Silver, S. & Roizman, B. (1985). Herpes simplex virus I mutant deleted in the α22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice. *J. Virol.* 55, 338-346.

Shepard, A. A., Imbalzano, A. N. & DeLuca, N. A. (1989). Separation of primary structural components conferring autoregulation, transactivation, and DNA-binding properties to the herpes simplex virus transcriptional regulatory protein ICP4. *J. Virol.* 63, 3714-3728.

Shieh, M.-T., WuDunn, D., Montgomery, R. I., Esko, J. D. & Spear, P. G. (1992). Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J. Cell Biol.* 116, 1273-1281.

Ship, II, Brightman, V. J. & Laster, L. L. (1967). The patient with recurrent aphthous ulcers and the patient with recurrent herpes labialis: a study of two population samples. *J Am Dent Assoc* 75, 645-54.

Ship, II, Miller, M. F. & Ram, C. (1977). A retrospective study of recurrent herpes labialis (RHL) in a professional population, 1958-1971. *Oral Surg Oral Med Oral Pathol* 44, 723-30.

Shukla, D., Liu, J., Blaiklock, P., Shworak, N. W., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D. & Spear, P. G. (1999). A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell* 99, 13-22.

Shukla, D. & Spear, P. G. (2001). Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. *J. Clin. Invest.* 108, 503-10.

Simmons, A. (2002). Clinical manifestations and treatment considerations of herpes simplex virus infection. *J Infect Dis.* 15, 1-7.

Smibert, C. A., Johnson, D. C. & Smiley, J. R. (1992). Identification and characterization of the virion-induced host shutoff product of herpes simplex virus gene UL41. *J. Gen. Virol.* 73, 467-470.

Smibert, C. A., Popova, B., Xiao, P., Capone, J. P. & Smiley, J. R. (1994). Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs. *J. Virol* 68, 2339-2346.

Smibert, C. A. & Smiley, J. R. (1990). Differential regulation of endogenous and transduced β-globin genes during infection of erythroid cells in herpes simplex virus type 1 recombinant. *J. Virol.* 64, 3882-3894.

Smiley, J. R., Duncan, J. & Howes, M. (1990). Sequence requirements for DNA rearrangements induced by the terminal repeat of herpes simplex virus type 1 KOS DNA. *Journal of Virology* 64, 5036-5050.

Smiley, J. R., Elgadi, M. M. & Saffran, H. A. (2001). Herpes simplex virus vhs protein. *Methods Enzymol.* 342, 440-51.

Smiley, J. R., Johnson, D. C., Pizer, L. I. & Everett, R. D. (1992). The ICP4 binding sites in the herpes simplex virus type 1 glycoprotein (gD) promoter are not essential for efficient gD transcription during virus infection. *J. Virol.* 66, 623-631.

Smith, C. A., Bates, P., Rivera-Gonzalez, R., Gu, B. & DeLuca, N. A. (1993). ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB. *J. Virol.* 67, 4676-4687.

Smith, J. S. & Robinson, N. J. (2002). Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review. *J Infect Dis.* 15, S3-28.

Sodeik, B., Ebersold, M. W. & Helenius, A. (1997). Microtuble-mediated transport of incoming hepres simplex virus 1 capsids to the nucleus. *J. Cell Biol.* 136, 1007-1021.

Soliman, T. M., Sandri-Goldin, R. M. & Silverstein, S. J. (1997). Shuttling of the herpes simplex virus type 1 regulatory protein ICP27 between the nucleus and cytoplasm mediates the expression of late proteins. *J. Virol.* 71, 9188-9197.

Soliman, T. M. & Silverstein, S. J. (2000). Herpesvirus mRNAs are sorted for export via Crm1-dependent and -independent pathways. *J Virol* 74, 2814-25.

Song, B., Yeh, K. C., Liu, J. & Knipe, D. M. (2001). Herpes simplex virus gene products required for viral inhibition of expression of G1-phase functions. *Virology* 290, 320-8.

Sorenson, C. M., Hart, P. A. & Ross, J. (1991). Analysis of herpes simplex virus-induced mRNA destablizing activity using an in vitro mRNA decay system. *Nucleic Acids Res.* 19, 4459-4465.

Spear, P. G. (1993). Entry of alphaherpesviruses into cells. *Semin. Virol.* 4, 167-180.

Spencer, C. A., Dahmus, M. E. & Rice, S. A. (1997). Repression of host RNA polymerase II transcription by herpes simplex virus type 1. *J. Virol.* 71, 2031-2040.

Stadler, M., Chelbi-Alix, M. K., Koken, M. H., Venturini, L., Lee, C., Saib, A., Quignon, F., Pelicano, L., Guillemin, M. C., Schindler, C. & et al. (1995). Transcriptional induction of the PML growth suppressor gene by interferons is mediated through an ISRE and a GAS element. *Oncogene* 11, 2565-73.

Stern, S. & Herr, W. (1991). The herpes simplex virus trans-activator VP16 recognized the Oct-1 homeo domain: evidence for a homeo domain recognition subdomain. *Genes. Dev.* 5, 2555-2566.

Stern, S., Tanaka, M. & Herr, W. (1989). The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. *Nature* 341, 624-30.

Stow, N. D. & Stow, E. C. (1986). Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate-early polypeptide Vmw110. *J. Gen. Virol.* 67, 2571-2585.

Strelow, L., Smith, T. & Leib, D. (1997). The virion host shutoff function of herpes simplex virus type 1 plays a role in corneal invasion and functions independently of the cell cycle. *Virology* 231, 28-34.

Strelow, L. I. & Leib, D. A. (1995). Role of the virion host shutoff (vhs) of herpes simplex virus type 1 in latency and pathogenesis. *J Virol* 69, 6779-86.

Strelow, L. I. & Leib, D. A. (1996). Analysis of conserved domains of UL41 of herpes simplex virus type 1 in virion host shutoff and pathogenesis. *J. Virol.* 70, 5665-7.

Stringer, K. F., Ingles, C. J. & Greenblatt, J. (1990). Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. *Nature* 345, 783-786.

Strom, T. & Frenkel, N. (1987). Effects of herpes simplex virus on mRNA stability. *J. Virol.* 61, 2198-2207.

Suzutani, T., Nagamine, M., Shibaki, T., Ogasawara, M., Yoshida, I., Daikoku, T., Nishiyama, Y. & Azuma, M. (2000). The role of the UL41 gene of herpes simplex virus type 1 in evasion of non-specific host defence mechanisms during primary infection. *J. Gen. Virol.* 81, 1763-71.

Svennerholm, B., Jeansson, S., Vahlne, A. & Lycke, E. (1991). Involvement of glycoprotein C (gC) in adsoprtion of herpes simplex virus type 1 (HSV-1) to the cell. *Arch. Virol.* 120, 273-279.

Sydiskis, R. J. & Roizman, B. (1966). Polysomes and protein synthesis in cells infected with a DNA virus. *Science* 153, 76-78.

Sydiskis, R. J. & Roizman, B. (1967). The disaggregation of host polyribosomes in productive and abortive infection with HSV. *Virology* 32, 678-686.

Sydiskis, R. J. & Roizman, B. (1968). The sedimentation profiles of cytoplasmic polyribosomes in mammalian cells productively and abortively infected with HSV. *Virology* 34, 562-565.

Taddeo, B., Esclatine, A., Zhang, W. & Roizman, B. (2003). The stress-inducible immediate-early responsive gene IEX-1 is activated in cells infected with herpes simplex virus 1, but several viral mechanisms, including 3' degradation of its RNA, preclude expression of the gene. *J Virol* 77, 6178-87.

Tal-Singer, R., Peng, C., Ponce de Leon, M., Abrams, W. R., Banfield, B. W., Tufaro, F., Cohen, G. H. & Eisenberg, R. J. (1995). Interaction of herpes simplex virus glycoprotein C with mammalian cell surface molecules. *J. Virol.* 69, 4471-4483.

Tang, Q., Li, L., Ishov, A. M., Revol, V., Epstein, A. L. & Maul, G. G. (2003). Determination of minimum herpes simplex virus type 1 components necessary to localize transcriptionally active DNA to ND10. *J Virol* 77, 5821-8.

Tatman, J. D., Preston, V. G., Nicholson, P., Elliott, R. M. & Rixon, F. J. (1994). Assembly of herpes simplex virus type 1 capsids using a panel of recombinant baculoviruses. *J. Gen. Virol.* 75, 1101-13.

Tatusova, T. A. & Madden, T. L. (1999). BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol Lett* 174, 247-50.

Tigges, M. A., Leng, S., Johnson, D. C. & Burke, R. L. (1996). Human herpes simplex virus (HSV)-specific CD8+ CTL clones recognize HSV- 2-infected fibroblasts after treatment with IFN-gamma or when virion host shutoff functions are disabled. *J. Immunol.* 156, 3901-10.

Tom, S., Henricksen, L. A. & Bambara, R. A. (2000). Mechanism whereby proliferating cell nuclear antigen stimulates flap endonuclease 1. *J Biol Chem* 275, 10498-505.

Trgovcich, J., Johnson, D. & Roizman, B. (2002). Cell surface major histocompatibility complex class II proteins are regulated by the products of the gamma(1)34.5 and U(L)41 genes of herpes simplex virus 1. *J. Virol.* 76, 6974-86.

Triezenberg, S. J., Kingsbury, R. C. & McKnight, S. L. (1988a). Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev.* 2, 718-729.

Triezenberg, S. J., LaMarco, K. L. & McKnight, S. L. (1988b). Evidence of DNA: protein interactions that mediate HSV-1 immediate early gene activation by VP16. *Genes Dev.* 2, 730-742.

Uprichard, S. L. & Knipe, D. M. (1996). Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes. *J. Virol.* 70, 1969-1980.

Van Sant, C., Hagglund, R., Lopez, P. & Roizman, B. (2001). The infected cell protein 0 of herpes simplex virus 1 dynamically interacts with proteasomes, binds and activates the cdc34 E2 ubiquitin-conjugating enzyme, and possesses in vitro E3 ubiquitin ligase activity. *Proc Natl Acad Sci U S A* 98, 8815-20.

Varmuza, S. L. & Smiley, J. R. (1985). Signals for site-specific cleavage o fHSV DNA: maturation involves two separate cleavages events at sites distal to the recognition sequences. *Cell* 41, 793-802.

Vogel, J. L. & Kristie, T. M. (2000). The novel coactivator C1 (HCF) coordinates multiprotein enhancer formation and
mediates transcription activation by GABP. *EMBO J.* 19, 683-90.

Wagner, E. K., Guzowski, J. F. & Singh, J. (1995). Transcription of the herpes simplex virus genome during productive and latent infection. *Prog. Nuc. Acid Res. Mol. Biol.* 51, 123-165.

Warner, M. S., Geraghty, R. J., Martinez, W. M., Montgomery, R. I., Whitbeck, J. C., Xu, R., Eisenberg, R. J., Cohen, G. H. & Spear, P. G. (1998). A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. *Virology* 246, 179-89.

Weir, J. P. (2001). Regulation of herpes simplex virus gene expression. *Gene* 271, 117-30.

Werstuck, G. & Capone, J. P. (1989a). Idenification of a domain of the herpes simplex virus trans-activator Vmw65 required for protien-DNA complex formation through the use of protein A fusion proteins. *J. Virol.* 68, 5509-5513.

Werstuck, G. & Capone, J. P. (1989b). Mutational analysis of the herpes simplex virus trans-inducing factor Vmw65. *Gene* 75, 213-224.

Whitley, R. J. (1996). Herpes simplex viruses. In *Fields Virology*, 3rd edn. Edited by B. N. Fields, D. N. Knipe, P. M. Howely & e. al. Philidelphia: Lippincott-Raven Publishers.

Whitley, R. J. & Gnann, J. W. (1993). The epidemiology and clinical manifestations of herpes simplex virus infections. In *The human herpesviruses*, pp. 69-105. Edited by B. Roizman, R. J. Whitley & C. Lopez. New York: Raven Press.

Williams, R. K. & Straus, S. E. (1997). Specificity and affinity of binding of herpes simplex virus type 2 glycoprotein B to glycosaminoglycans. *J. Virol.* 71, 1375-80.

Wilson, J. E., Pestova, T. V., Hellen, C. U. & Sarnow, P. (2000). Initiation of protein synthesis from the A site of the ribosome. *Cell* 102, 511-20.

Witherell, G. W., Schultz-Witherell, C. S. & Wimmer, E. (1995). Cis-acting elements of the encephalomyocarditis virus internal ribosomal entry site. *Virology* 214, 660-3.

Wu, C. A., Nelson, N. J., McGeoch, D. J. & Challberg, M. D. (1988). Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. *J. Virol.* 62, 435-443.

Wu, C. L. & Wilcox, K. W. (1990). Codons 262 to 490 from the herpes simplex virus ICP4 gene are sufficient to encode a sequence-specific DNA binding protein. *Nucleic Acids Res.* 18, 531-538.

WuDunn, D. & Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* 63, 52-58.

Xia, K., DeLuca, N. A. & Knipe, D. M. (1996). Analysis of phosphorylation sites of herpes simplex virus type 1 ICP4. *J Virol* 70, 1061-71.

Xiao, H., Pearson, A., Coulombe, B., Truant, R., Zhang, S., Regier, J., Triezenberg, S., Reinberg, D., Flores, O., Ingles, C. & Greenblatt, J. (1994). Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. *Mol. Cell Biol.* 14, 7013-7024.

Xiao, P. & Capone, J. P. (1990). A cellular factor binds to the herpes simplex virus type 1 transactivator Vmw65 and is required for Vmw65-dependent protein-DNA complex assembly with Oct-1. *Mol. Cell. Biol.* 10, 4974-4977.

Xiao, W., Pizer, L. I. & Wilcox, K. W. (1997). Identification of a promoter-specific transactivation domain in the herpes simplex virus regulatory protein ICP4. *J Virol* 71, 1757-65.

Yao, F. & Schaffer, P. (1994). Physical interaction between the herpes simplex virus type 1 immediate-early regulatory proteins ICP0 and ICP4. *J. Virol.* 68, 8158-8168.

Ye, G. J., Vaughan, K. T., Vallee, R. B. & Roizman, B. (2000). The herpes simplex virus 1 U(L)34 protein interacts with a cytoplasmic dynein intermediate chain and targets nuclear membrane. *J Virol* 74, 1355-63.

Yeh, L. & Schaffer, P. A. (1993). A novel class of transcripts expressed with late kinetics in the absence of ICP4 spans the junction between the long and short segments of the herpes simplex virus type 1 genome. *J. Virol.* 67, 7373-7382.

York, I., Roop, C., Andrews, D. W., Riddel, S. R., Graham, F. L. & Johnson, D. C. (1994). A cytosolic herpes simplex virus protein inhibits antigen presentation to $CD8^-$ T lymphocytes. *Cell* 77, 525-535.

Zelus, B. D., Stewart, R. S. & Ross, J. (1996). The virion host shutoff protein of herpes simplex virus type 1: messenger ribonucleolytic activity in vitro. *J. Virol.* 70, 2411-2419.

Zhou, C. & Knipe, D. M. (2002). Association of herpes simplex virus type 1 ICP8 and ICP27 proteins with cellular RNA polymerase II holoenzyme. *J Virol* 76, 5893-904.

Zhou, Z. H., Chen, D. H., Jakana, J., Rixon, F. J. & Chiu, W. (1999). Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. *J Virol.* 73, 3210-8.